Evaluation of Microbial Dynamics on Low-Sodium Cooked Bologna Under Different Packaging Conditions

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By

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ABSTRACT

The purpose of this study was to assess the growth of spoilage and pathogenic bacteria on low sodium concentration sliced cooked bologna under refrigerated storage conditions. In study 1, the effect of three different sodium concentrations (1%, 2% and 3%) and two packaging conditions (aerobic and vacuum) on growth of a cocktail of inoculated spoilage bacteria such *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Brochothrix thermosphacta* and *Pseudomonas fluorescens* were investigated by using culture-dependent and culture-independent techniques. In general, reducing the sodium concentration from 2% (the current industry standard) to 1% NaCl in the cooked bologna system did not have a significant effect on microbial growth.

The utilization of Ion Torrent high-throughput sequencing in this study allowed the description of the total microbial community present on sliced cooked bologna. Taxonomic analysis revealed the microbial community belongs to the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes.*

Finally, in this study, *in situ* meat redox measurement values were collected over time using platinum electrodes placed on top of, and in between bologna slices. While the redox values obtained were, in general, consistent with increasing bacterial cell numbers, issues of reproducibility and consistency were evident.

The second study focused on the impact that the addition of a bacteriocin, Micocin X®, to the meat blend would have on growth of spoilage bacteria and in particular, *Listeria monocytogenes*, in vacuum-packaged cooked bologna formulated with 1% and 3% NaCl. In general, results demonstrated Micocin X® had a significant effect on the growth of *Pseudomonas fluorescens* and *Listeria monocytogenes;* however, no substantial effect was assessed in the control of lactic acid bacteria or *Brochothrix thermosphacta*.

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TABLE OF CONTENTS

PERMISSION TO USE i

ABSTRACT ii

ACKNOWLEDGMENTS iii

TABLE OF CONTENTS iv

LIST OF FIGURES viii

LIST OF TABLES x

1. INTRODUCTION 1

1.1. General 1

1.2. Hypotheses 2

1.3. Objectives 2

2. LITERATURE REVIEW 3

2.1. Meat quality 3

2.2. Meat shelf-life 3

2.3. Function of salt in meat products 3

2.4. Physical processing effects 3

2.5. Sensory properties 4

2.6. Preservative effect 4

2.7. Health effects of salt 4

2.8. Sources of sodium from meat products 5

2.9. Processed meat 5

2.9.1. Meat curing ingredients 5

2.9.2. Nitrite/nitrate 5

2.9.3. Sodium ascorbate or erythorbate 6

2.9.4. Phosphates 7

2.9.5. Sweeteners 7
2.9.6. Binders and extenders 7
2.10. Reduction of sodium in processed meat 7
2.11. Approaches to salt reduction in meat products 8
2.12. Factors that influence microbial growth 9
2.13. Packaging techniques 10
2.13.1. Vacuum packaging 11
2.13.2. Modified atmosphere packaging 11
2.14. Microflora of various ready-to-eat meat products 12
2.14.1. Fermented products 13
2.14.2. Cured hams 14
2.14.3. Cooked meat 14
2.15. Characterization of microbial communities 15
2.15.1. Culture-dependent methods 15
2.15.2. Culture-independent methods 17
2.16. Bacteriocins 19
2.16.1. Bacteriocin classification and mode of action 19
2.16.2. Mechanisms of resistance to bacteriocins 20
2.17. Biopreservation 20

3. INVESTIGATION OF LOW-SODIUM SLICED COOKED BOLOGNA QUALITY USING CULTURE-DEPENDENT AND CULTURE-INDEPENDENT TECHNIQUES 23
3.1. Abstract 23
3.2. Introduction 24
3.3. Material and Methods 25
3.3.1. Experimental design 25
3.3.2. Meat handling 27
3.3.3. Manufacture of cooked bologna 27
3.3.4. Bacteria and preparation of inocula 29
3.3.5. Inoculation procedure 30
3.3.6. Microbial analyses 30
3.3.7. Turbidimetric assay 31

3.3.8. Water activity (a_w) 31
3.3.9. pH measurements. 32
3.3.10. Redox potential monitoring 32
3.3.11. DNA extraction and PCR amplification 32
3.3.12. Ion Torrent 16S rRNA gene sequencing 33
3.3.13. Statistical analysis 34
3.4. Results and Discussion 34
3.4.1. Optimization of the experiment results 34
3.4.1.1. Microbiological analysis 34
3.4.1.2. Chemical analysis 35
3.4.1.3. Ion Torrent analysis 35
3.4.1.4. Turbidimetric assay 37
3.4.2. Enumeration of *Brochothrix thermosphacta* and LAB 39
3.4.3. Enumeration of *Pseudomonas fluorescens* 43
3.4.4. Culture-independent community identification 48
3.4.5. Chemical analyses 55
3.4.5.1. pH and water activity 55
3.4.5.2. Redox potential 55
3.5. Conclusions 57
3.6. Connection to the next study 59

4. SIMULATED POST-PROCESSING CONTAMINATION OF LOW SALT SLICED COOKED BOLOGNA WITH *LISTERIA MONOCYTOGENES*, PRODUCED WITH AND WITHOUT MICOCIN X® 61
4.1. Abstract 61
4.2. Introduction 62
4.3. Materials and Methods 63
4.3.1. Experimental design 63
4.3.2. Manufacture of cooked bologna 63
4.3.3. Bacteria and preparation of inoculum 64
4.3.4. Inoculation procedure 65
4.3.5. Microbial analysis 65

4.3.6. Water activity (A_w) 66
4.3.7. pH measurements. 66
4.3.8. DNA extraction and PCR amplification 66
4.3.9. Ion Torrent 16S rRNA gene sequencing 66
4.3.10. Statistical analysis 67
4.4. Results and Discussion 67
4.4.1. Enumeration of LAB 67
4.4.2. Enumeration of *Brochothrix thermosphacta* 69
4.4.3. Enumeration of *Pseudomonas fluorescens* 70
4.4.4. Enumeration of *Listeria monocytogenes* 72
4.4.5. Chemical analyses 75
4.4.6. Bacterial community determined by culture-independent technique 76
4.5. Conclusions 81
5. GENERAL DISCUSSION 82
6. GENERAL CONCLUSIONS 85
7. REFERENCES 87

LIST OF FIGURES

Figure 3.1 Schematic diagram illustrating the experimental procedure conducted over 40 days during Trial 1 and over 30 days during Trial 2 at a temperature of 4°C……...……26

Figure 3.2 Diagram illustrating the analysis of data of Study 1. 28

Figure 3.3 Growth of LAB, *P. fluorescens* and *B. thermosphacta* on sliced cooked bologna formulated with 1, 2 and 3% NaCl and stored under aerobic and vacuum-packaged conditions over 12 days at 8°C. 36

Figure 3.4 Ion Torrent sequence data showing frequency (%) of genera detected. 37

Figure 3.5 Growth response of LAB and *B. thermosphacta* on sliced cooked bologna 1, 2 and 3% NaCl under aerobic conditions over 30 d at 4°C. 40

Figure 3.6 Growth response of LAB and *B. thermosphacta* on sliced cooked bologna with 1, 2 and 3% NaCl under vacuum conditions 30 d at 4°C 44

Figure 3.7 Growth response of *P. fluorescens* on Trial 1 and Trial 2 on sliced cooked bologna with 1, 2 and 3% NaCl under aerobic conditions 30 d at 4°C. 46

Figure 3.8 Growth response of *P. fluorescens* on Trial 1 and Trial 2 on sliced cooked bologna with 1, 2 and 3% NaCl under vacuum conditions at 4°C. 48

Figure 3.9 Taxonomic classification of bacterial reads at phylum level. 52

Figure 3.10 Taxonomic classification of bacterial reads at order level. 54

Figure 3.11 Effect of storage and NaCl concentration on redox potential (Eh) measurements of sliced cooked bologna at the slice surface, and between slices, for 30 days on aerobic and vacuum-packaged product on bologna product formulated with 1% and 3% NaCl**.** 58

Figure 4.1 Schematic diagram illustrating the experimental procedure conducted over 30 days during Trial 1 and Trial 2 at a temperature of 4°C.. 64

Figure 4.2 Diagram illustrating data analysis of data Study 2. 65

Figure 4.3 Numbers of viable LAB cells on sliced cooked, vacuum-packaged bologna formulated with or without Micocin X® and with 1% NaCl or 3% NaCl in the presence and absence of *Listeria monocytogenes* stored at 4°C. 68

Figure 4.4 Numbers of viable *Brochothrix thermosphacta* cells on sliced cooked, vacuum-packaged bologna formulated with or without Micocin X® and with 1% NaCl or 3% NaCl in the presence and absence of *Listeria monocytogenes* stored at 4°C.. 70

Figure 4.5 Numbers of viable *Pseudomonas fluorescens* cells on sliced cooked, vacuum-packaged bologna formulated with or without Micocin X® and with 1% NaCl or 3% NaCl in the presence and absence of *Listeria monocytogenes* stored at 4°C.. 72

Figure 4.6 Numbers of viable *Listeria monocytogenes* on sliced cooked, vacuum-packaged bologna during Trial 1 and Trial 2. On meat formulated with or without Micocin X® in the presence and absence of spoilage bacteria stored at 4°C. 74

Figure 4.7 Taxonomic classification of bacterial reads at phylum level 77

Figure 4.8 Taxonomic classification of bacterial reads at order level 80

LIST OF TABLES

Table 2.1 Gas mixture recommended for modified atmosphere packaging of meat products ... 12

Table 2.2 Barrier properties of various plastic material commonly used in meat packaging 13

Table 3.1 Bologna formulations (%, w/w) incorporating three different levels of NaCl 28

Table 3.2 Proximate composition of cooked bologna formulated with three different levels of NaCl 30

Table 3.3 Maximum specific growth rates (μ_{max}; hr^{-1}) estimated for different bacteria as a function of the final salt concentration of 1, 2 and 3% (NaCl) at 15°C 38

Table 3.4 Doubling times for different bacteria in broth formulated with a final concentration of 1, 2 and 3% NaCl at 15°C. 39

Table 3.5 Viable counts of spoilage bacteria on sliced cooked bologna stored under aerobic or vacuum-packaged conditions at 4°C 40

Table 3.6 P-value for estimated parameters for LAB and *B. thermosphacta* growth in sliced cooked bologna 43

Table 3.7 P-value for estimated parameters for *Pseudomonas fluorescens* growth in sliced cooked bologna 45

Table 3.8 Effect of sodium concentration on water activity 55

Table 4.1 Bologna formulation (%, w/w) with two different NaCl levels, with and without Micocin X® 66

Table 4.2 P-value for estimated parameters for LAB growth in combination with *Listeria monocytogenes* in vacuum-packaged sliced cooked bologna 69

Table 4.3 P-value for estimated parameters for *Brochothrix thermosphacta* growth in absence and presence of *L. monocytogenes* in vacuum-packaged sliced cooked bologna 69

Table 4.4 P-value for estimated parameters for *Pseudomonas fluorescens* growth in absence and presence of *L. monocytogenes* in vacuum-packaged sliced cooked bologna 71

Table 4.5 Differences of least square means for growth of *Pseudomonas fluorescens* at 1 and 3% NaCl in the presence and absence of Micocin X®. 71

Table 4.6 P-value for estimated parameters for *Listeria monocytogenes* growth in absence and presence of spoilage bacteria in vacuum-packaged sliced cooked bologna. 75

Table 4.7 Number of sequences observed and analyzed for 16s rRNA of bologna samples formulated with two sodium concentrations with or without bacteriocin. 76

1. INTRODUCTION

1.1. General

Sodium is an essential nutrient in the human diet, but its excessive intake has been linked to the development of hypertension, cardiovascular disease and kidney failure. A high-sodium diet thus represents a serious population health risk according to many organizations, including the World Health Organization, Health Canada and the United States Department of Agriculture (USDA). Canadians between one and 70 years of age consume twice as much sodium (9 to 12 g/day) as considered to be adequate (1 to 2 g/day) (CTAC 2009; 2010).

Approximately 75% of the salt consumed is derived from processed foods in the form of sodium chloride (Aaslyng *et al.*, 2014). The food industry plays an important role in the strategy to reduce salt intake per day in the Canadian population. Therefore, the food industry faces the challenge of producing food products low in salt without compromising taste, texture and appearance. Also, reducing salt levels below those typically used without adding any other preservative has the potential to influence microbial stability, thereby affecting the product's shelf life and safety.

Since water activity is one of the most important hurdles used in food preservation, other factors that control the growth of spoilage organisms must be used when salt concentrations are changed. Lactic acid bacteria and their metabolites have potential as an antimicrobial hurdle because they are generally recognized as safe (GRAS) substances; they are usually pH- and heat-tolerant and they have a relatively broad antimicrobial spectrum. Micocin®, a biopreservative that contains three bacteriocins produced by the lactic acid bacterium C*arnobacterium maltaromaticum* CB1, has been tested and recently approved (2010) in Canada, USA, Mexico, Central America, Caribbean and Colombia as a food additive with antimicrobial effect.

The aim of this thesis was to follow the dynamics of spoilage and pathogenic microorganisms in low sodium, sliced, cooked bologna product by molecular and traditional

culture-based methods. Understanding the microbial ecology and behavior will enable better control of the spoilage process, improving the safety and competitiveness in low sodium products.

1.2. Hypotheses

The following hypotheses were tested during this study:

1. Changes in sodium concentration will affect microbial growth kinetics. Specifically, sliced cooked bologna with reduced NaCl concentrations (1 or 2%) will have a shorter microbial shelf life in comparison with a product formulated with 3% NaCl.
2. Reducing sodium content in sliced cooked bologna will promote the growth of *Listeria monocytogenes*. Therefore, the addition of Micocin X®, a bacteriocin, as a preservative will decrease the number of viable *L. monocytogenes* cells, along with the general spoilage microflora, regardless of sodium concentration.

1.3. Objectives

The main objective of this study was to investigate the microbial shelf life of low sodium sliced cooked bologna by revealing microbial diversity and dynamic changes (patterns and magnitudes of change) occurring during refrigerated storage, and to investigate the efficacy of Micocin X® as an antimicrobial agent in the inhibition of spoilage and pathogenic microorganism in this low salt processed product.

In order to accomplish these objectives, some technical considerations were identified:

1. To determine the biodiversity and relative abundances of spoilage microflora associated with the low-salt meat model product during storage at refrigerated temperature using culture-dependent and culture-independent techniques, including Ion Torrent sequencing, and

2. To inoculate the low-salt meat product model with dominant spoilage bacteria used in objective #1 along with the pathogen *L. monocytogenes* and thereafter monitor, using conventional culture techniques and DNA sequencing methods, the effect of salt alone, as well as in combination with Micocin X®.

2. LITERATURE REVIEW

2.1. Meat quality

Meat quality is a common term used to describe properties of meat and consumers' perceptions when buying food. This term includes characteristics such as appearance, texture, juiciness, shelf life and microbiological, chemical and physical safety (Lawrie, 1991; Aberle *et al.*, 2001).

2.2. Meat shelf-life

Shelf-life is defined as the length of time for which a product can be stored under specific conditions while maintaining acceptable quality. Product shelf-life can be extended by inhibiting or retarding the growth of undesirable microflora, which can be achieved by the manipulation of the meat microenvironment.

The shelf-life of fresh meats can be determined based on the total viable count of bacteria (TVC), which is expressed as the number of organisms present per gram of meat product. The commercial shelf-life of cooked, vacuum-packaged meat products is largely dependent on the presence of lactic acid bacteria, which causes souring, slimy meat, and swelling of the pack due to gas production (Kreyenschmidt *et al.*, 2010). The shelf-life of cooked ham products has been reported to be up to 30 days at 1-8 °C (Vercammen *et al*., 2011); cooked ham is considered spoiled when bacterial numbers are 10^6/g or higher (Kreyenschmidt *et al.*, 2010).

2.3. Function of salt in meat products

Salt is a key ingredient used during the processing of meat products (Aberle *et al.*, 2001). It is added to foods to prevent microbial growth, to obtain the desired texture and to enhance meat taste and flavour (Weiss *et al.*, 2010).

2.4. Physical processing effects

Salts aid in solubilization of myofibrillar proteins. The effect of NaCl on meat proteins is most likely caused by the fact that Cl^- ions form a stronger bond with the positively-charged groups of myosin or actomyosin than do the Na^+ ions (Desmond, 2006), resulting in an increase

in negative charges of proteins thereby increasing repulsion between them. Therefore, an increase of swelling of myofibrillar tissue occurs (Hamm, 1986), as well as an overall improvement in the water and fat binding ability of meat, which results in the formation of a gel texture upon cooking along with increased product yield (Aberle *et al.*, 2001; Desmond, 2006).

2.5. Sensory properties

Salt is added to a wide range of processed foods for its effects on taste and flavour. It affects flavour by providing saltiness, by enhancing and modifying the flavour of other ingredients, and by controlling growth of microbes that produce compounds that can alter the flavour of the product (Doyle and Glass, 2010).

2.6. Preservative effect

Salt contributes, along with other preservatives and processing, to the prevention of the spoilage of foods and the extension of the shelf-life of the product (Aberle *et al.*, 2001; Weiss *et al*., 2010). The mechanism by which this is achieved is through the reduction of water activity (a_w) and by affecting the osmotic pressure of the environment. However, it is important to note that the microbiological stability of a product also depends on other key factors such as pH and temperature (Weiss *et al*., 2010).

Responses to salt concentration among microorganisms vary with the species. For example, *Campylobacter* spp. are sensitive to a salt concentration of 0.5%, *Clostridium botulinum* can grow in 10% NaCl and *Staphylococcus aureus* can grow in the presence of 20% salt (Doyle and Glass, 2010). On the other hand, using COMBASE (a database used for quantitative and predictive food microbiology), it has been estimated that *Pseudomonas* sp. is able to growth in broth culture with 6.0 % NaCl, pH 6 at 10°C; whereas, *Brochothrix* sp. is able to multiply at a maximum NaCl concentration of 7.5%, pH 6 and a temperature of 10°C (Doyle and Glass, 2010).

2.7. Health effects of salt

Several studies (Meneton *et al.*, 2005; Rodriguez-Iturbe *et al.*, 2007; Marketou *et al.*, 2013) have shown a direct relationship between the excessive intake of sodium and increased incidence of hypertension. The kidney participates in the regulatory mechanisms of blood pressure. When sodium intake is higher, kidney function may decline affecting the homeostatic regulation of electrolytes. As the efficiency of excretion of excess sodium decreases, plasma volume may

increase, thereby stressing the cardiovascular system by inducing hypertension (Doyle and Glass, 2010; Weiss *et al.*, 2010).

Hypertension is recognized to be a risk factor for cardiovascular disease and is often associated with other cardiovascular risk factors, such as obesity and elevated blood lipids, in a condition called 'metabolic syndrome'. Some studies have also suggested that high dietary sodium levels are associated with other health issues like the development of bone disease in which the retention of calcium is affected by high concentrations of sodium, altering of calcium retention and bone density (Cohen and Roe, 2000; Doyle and Glass, 2010).

2.8. Sources of sodium from meat products

The main type of sodium that is added during processing in meat products is sodium chloride, which has a sodium content of 39.3% (Ruusunen and Puolanne, 2005). Sodium is also a constituent of many other additives used in meat processing. For example, the concentration of sodium in sodium tripolyphosphate is 31.2%, 27.1% in sodium nitrate, 11.6% in sodium erythorbate, and 33.2% in sodium nitrite (Desmond, 2006).

2.9. Processed meat

Processed meats are products where fresh meat properties have been changed by the use of one or more procedures like chopping, seasoning or heat treatment (Aberle *et al*., 2001). The processed meat category includes products such as ham, bacon, smoked meat, marinated products, and dry or fermented sausages.

2.9.1. Meat curing ingredients

Salt and nitrite are the two main ingredients often added in order to cure meats (Pegg, 2004; Shahidi *et al*., 2004). Sodium chloride is included in all meat curing formulae due to the characteristics listed above.

2.9.2. Nitrite/nitrate

Nitrite has different functions during meat preservation and is responsible for the development of the specific cured flavour and colour of processed meats. Nitrate must first be reduced to nitrite by microorganisms to cause color change. When nitrite is added to water, it forms nitrous acid and nitric oxide. Nitric oxide then combines with myoglobin forming a bright

red color called nitric oxide myoglobin. A change in colour and stability then occurs upon heat denaturation of the protein portion of myoglobin. The resulting pigment is nitrosylhemochromogen, and is responsible for the bright pink color characteristic of cured meats (Aberle *et al*., 2001; Weiss *et al.,* 2010).

Nitrite also has antioxidant effects that prevent lipid oxidation as well as the formation of warmed-over flavour in cured meat products. Finally, this compound acts as an antimicrobial agent, retarding the growth of key microorganisms such as C*lostridium botulinum,* with particular importance in the prevention of germination of spores and subsequent production of botulinum toxin (Aberle *et al*., 2001).

High nitrite concentrations favors lactic acid bacteria growth, and the pronounced inhibitory effect of sodium nitrite has been observed at a high concentration of 400 ppm (Korkeala *et al.,* 1992). Low nitrite levels may allow growth of *Brochothrix thermosphacta*. In a study conducted by Nielsen (1983) it was shown that *Brochothrix thermosphacta* was able to grow on vacuum-packaged cooked bologna with 100 ppm nitrite added when stored at a temperature of 5°C and 2°C, reaching counts of 10^6 CFU/g in 10 and 16 days, respectively. On the other hand, when a nitrite concentration of 200 ppm was used in the formulation, there was no development of *B. thermosphacta* over a 5 weeks storage period.

Due to the potential formation of carcinogenic compounds such as nitrosamines from nitrites and amine compounds, Canadian regulations allow only 200 ppm nitrite to be used in hams and 120 ppm nitrite in bacon (Canadian Food Inspection Agency, 2011).

2.9.3. Sodium ascorbate or erythorbate

Sodium erythorbate and ascorbate are two common reducing agents added to the curing brine in order to speed the reduction of metmyoglobin to myoglobin, as well as the reduction of nitrite to nitric oxide. In addition, residual erythorbate and ascorbate help prevent the formation of carcinogenic *N*-nitrosamines (Pegg, 2004) and add stability to the cured meat pigment by reducing the deterioration of the nitrosohemochrome (Aberle *et al*., 2001). In Canada, there are no specific regulations for the use of these reducing agents in meat products. Rather, the allowable levels are determined as specified by Good Manufacturing Practices (Department of Justice Canada, 2011), and are around 500 ppm.

2.9.4. Phosphates

Phosphates are added to the cure or brine to increase the water-binding capacity and thereby reduce the shrinkage or purge of the finished product (Shahidi *et al*., 2004). Alkaline phosphates help to solubilize muscle proteins by increasing pH and by causing an increase in the number of positive charges on the proteins (Aberle *et al.,* 2001). Phosphates also are known to improve cured meat flavour by retarding oxidative rancidity and warmed-over flavour by chelating pro-oxidant metal ions (Pegg, 2004; Shahidi *et al*., 2004).

In Canada, the maximum level of phosphate salts that can be added to meat products is 0.5% of total added phosphate calculated as sodium phosphate dibasic (Canadian Food Inspection Agency, 2011).

2.9.5. Sweeteners

Sugar is added to cured meats primarily for flavour. Sweeteners used in meat products include sucrose, dextrose, corn syrup or corn syrup solids, and lactose (Pegg, 2004). During the cooking process, the sugars interact with the amino groups of protein to produce a desirable brown color and caramel flavour in some products, such as bacon, via the Maillard reaction. Sugar (usually dextrose) also provides substrate for the fermentative growth of various lactic acid-producing bacteria that provide the characteristic flavour and low pH of some dry cured and fermented sausage products (Aberle *et al*., 2001; Shahidi *et al*., 2004).

2.9.6. Binders and extenders

Binders and extenders are included in processed meat formulations in order to improve meat batter stability, water binding capacity, enhance texture or flavour and reduce shrinkage during cooking. Commonly-used extenders in meat formulations are generally high in protein content, and usually are either dried milk or soybean products in the USA (Aberle *et al*., 2001) and wheat flour in Canada.

2.10. Reduction of sodium in processed meat

It has been established that the consumption of more than 6 g NaCl/day/person is associated with the development of health problems such as elevated blood pressure. In Canada, efforts were initiated to reduce the per-person sodium intake by the year 2016 from 3,400 mg per day to a maximum of 2,300 mg per day and 1,500 mg of sodium per day for those aged 50 or older (CTAC, 2009-2010; Sodium Working Group, 2010). However, by the end of 2013, this

maximum sodium intake limit for those aged over 50 was revised by Hypertension Canada to no more than 2,000 mg/ per day.

Sodium reduction of meat products is highly-dependent on the type of product, its composition, and processing procedure (Ruusumen and Puolanne, 2005). For instance, it may not necessarily be possible to achieve these salt concentration targets in all processed foods.

Some studies have shown that the NaCl content of meat products such as bologna sausages and cooked hams can be lowered to 1.5 – 1.7%, and phosphate to 1.4%, without affecting the product's technological quality and yield. The recommended sodium content given for hams should be about 0.3% units higher than the NaCl content for cooked sausages due to the lower fat content (Ruusumen and Puolanne, 2005). Pappa *et al.* (2000) created satisfactory low-salt (1.3%) and low-fat (9.0%) frankfurters by using pectin to improve the product texture, yielding a product which was comparable to commercial products, while achieving a 48% lower salt content.

In fermented sausages, there currently is insufficient information available for reformulation, since salt is an essential ingredient of dry fermented sausages. However, Ruusumen and Puolanne (2005) concluded that the minimum NaCl concentration might be approximately 2.0%.

2.11. Approaches to salt reduction in meat products

A change in the concentration of sodium in foods, specifically in processed meats, may alter the shelf life of the product, as it reduces one of the key "hurdles" that control microbial growth. There are several studies that have suggested new alternatives that do not compromise the quality or texture of the product and most importantly, consumers' acceptance (Ruusumen and Puolanne, 2005).

The first approach (Desmond, 2006) involves replacement of NaCl with other chloride salts like KCl, $CaCl_2$ and $MgCl_2$. Potassium chloride is the most common salt substitute used, but it can give a bitter or sour taste to the product. Secondly, some studies have suggested replacing part of the NaCl with phosphates (Ruusunen and Puolanne, 2005). For example, the usage of sodium polyphosphate has been suggested by Desmond (2006), since it contains 31.24% sodium compared to 39.34% in NaCl and is used at 0.5% compared to a 2 – 4% usage rate for salt. Finally, the use of flavour enhancers that do not have a salty taste, but enhance the saltiness of products when used in combination with salt, may also be used. A mixture of mineral salt with

half of the sodium removed and replaced with magnesium sulphate and the amino acid L-lysine hydrochloride is another possibility (Ruusunen and Puolanne, 2005; Desmond, 2006).

2.12. Factors that influence microbial growth

The growth rate and survival of all microorganisms are influenced by the interaction of various extrinsic and intrinsic factors, such as acidity (pH), water activity (a_w) and redox potential.

2.12.1. Water activity

Water activity (a_w) is defined as the amount of water available for microbial growth and is based on a scale of 0 to 1.0, with pure water having a water activity of 1.00. When other growth conditions are near optimal values, the a_w required for the majority of microorganisms is in the range 0.995-0.980. For *Escherichia coli, Streptococcus faecalis,* and *Salmonella oranienburg* the a_w for growth ranges from 0.999 to 0.995. However, other microorganisms, such as *Pseudomonas fluorescens,* have lower water requirements (from 0.970 to 0.945); *Staphylococcus aureus* needs a minimum a_w of 0.86, *Listeria monocytogenes* requires an a_w of 0.92; whereas, lactic acid bacteria and *Brochothrix thermosphacta* need a minimum a_w of 0.94 (Blickstad, 1984; Christian, 2000; Doyle and Glass, 2010).

Manipulation of a_w is the method of preservation most commonly used in a wide range of foods to control a variety of spoilage and pathogenic bacteria. Meat products such as liver sausage, bologna and low salt-bacon have been reported to have a_w values ranging from 0.95 to 1.00 (Lund and Eklund, 2000).

2.12.2. pH

The pH of foods influences their susceptibility to microbial growth and acidification, and is used in the preservation of different type of foods. In their unprocessed state, most foods, such as meat, fish, and vegetables, are slightly acidic; most fruits are moderately acidic (Lund and Eklund, 2000).

It is well known that microorganisms have pH optimal, minimal, and maximal values. The optimum pH for growth of many food-associated microorganisms is in the range 6.5-7.5, but many organisms can multiply under acidic conditions. For example, *L. monocytogenes* requires a minimum pH of 4.39 for growth, has a pH optimum of 7.0, and cannot grow above pH 9.0; *P. fluorescens* requires a minimum pH of 4.4 for growth; whereas, *Lactobacillus curvatus* has a

minimum and a maximum pH of 3.0 and 9.8, respectively (Lund and Eklund, 2000; Lechiancole *et al.*, 2002).

In general, the inhibitory effect of pH on microbes is due to the metabolic costs of maintaining an intracellular pH in the near-neutral pH range. For instance, when the external pH is lowered, some decrease in the cell's cytoplasmic pH results; eventually, the difference between the internal and external pH exceeds the cell's ability to regulate the internal pH, at which point the cell dies.

As with other factors, pH usually interacts with other parameters in the food to inhibit microbial growth. Accordingly, pH interacts with factors such as a_w, salt, temperature, redox potential, and preservatives to inhibit growth of pathogens and other organisms.

2.12.3. Redox potential

The oxidation-reduction, or redox, potential (E_h) is a measurement, typically expressed in millivolts, that describes the oxidizing (electron accepting) or reducing (electron donating) potential of a substance (Morris, 2000). The possible relationship between microbial growth and E_h was first mentioned by Potter (1911) when he reported that a growing microbial culture created a more reducing environment when compared to the uninoculated medium. Thereafter, different investigations have reported the optimum E_h ranges in which different microorganisms can grow. For example, aerobes can grow over an Eh range of +500 to +300 mV, facultative anaerobes can grow over an E_h range of +300 to -100 mV, and anaerobes can grow from +100 to less than -250 mV (Morris, 2000). Strict anaerobes have ever more stringent E_h growth requirements.

Foods also have different redox potentials, the value of which is dependent upon the pH of the food, manner of processing, microbial growth, packaging and ingredients. In meat and meat products, cooked sausages and canned meats have a redox value in the range of 0 to 60 mV. It has been reported that the E_h of fresh horsemeat muscle fell from +250 to -130 mV immediately after death and that at on-set of rigor it was 0 to -50 mV. This change was believed to be due to enzymatic activity, oxygen depletion and pH reduction (Morris, 2000).

2.13. Packaging techniques

Packaging is the process or means by which a product is enclosed with protective material, whose function is to serve as a shield against hazards that may affect the quality of the raw and/or

cooked meat product by preventing microbial growth, chemical, and sensory changes during handling, distribution, and storage (Nychas *et al*., 2008; Chen *et al*., 2012). In the meat industry, vacuum-packaging and modified atmosphere packaging (MAP) are the packaging techniques most commonly used (Chen *et al*., 2012).

2.13.1. Vacuum packaging

Vacuum packaging is the method most commonly used for cooked meat products such as ham and sliced bologna (Kotzekidou and Bloukas, 1996; Lawrence and Mancini, 2008). This packaging technique consists on removing the air from a package and hermetically sealing it, reducing the O_2 level to less than 1%. (Argyri *et al.,* 2012). Food quality and shelf-life depend on the properties of the packaging used since it determines the type of microflora that will develop within the package due to the microenvironment created and due to the permeability to air and water vapour characteristics of the film used (Ščetar *et al*., 2010). Exclusion of oxygen in vacuum-packages reduces the growth of aerobic bacteria such as Gram-negative *Pseudomonas* species (Cayré *et al*., 2005). However, it is important to consider that *Pseudomonas* is known to have a high affinity for low partial pressures of oxygen and this spoilage bacterium can grow at relative low partial pressures of oxygen (Göran, 2000). On the other hand, vacuum packaging is still not efficient in inhibiting *Listeria monocytogenes,* which can survive and multiply under refrigerated vacuum packaging conditions, nor other microorganism such *Brochothrix thermosphacta* or lactic acid bacteria (Chen *et al*., 2012). In fact, lactic acid bacteria, including genera of *Lactobacillus*, *Carnobacterium*, and *Leuconostoc,* are normally known to increase in number and dominate the meat system under vacuum-packaging conditions (Nowak and Krysiak, 2005; Chen *et al*., 2012).

2.13.2. Modified atmosphere packaging

Modified atmosphere packaging (MAP) is the removal and/or replacement of the atmosphere surrounding the product before sealing in vapour-barrier materials (Phillips, 1996). In this technique, a gas mixture is usually flushed through the modified atmosphere, providing a hurdle condition that helps to restrict the growth of microorganisms. The main gases used in MAP are oxygen, nitrogen and carbon dioxide.

Carbon dioxide (CO_2) is included for its inhibitory effect. Nitrogen (N_2) is non-inhibitory, but because of its low solubility in water and fat, the presence of N_2 can delay oxidative rancidity

and prevent pack collapse that can occur when high concentrations of CO_2 are used. Low Oxygen (O_2) level is used to maintain the pigment myoglobin in its oxygenated form, oxymyoglobin. However, in cooked meats such as cured ham, low residual oxygen promotes pigment denaturation, for instance, oxygen scavengers are used (Walsh *et al.*, 2002; O'Grady and Kerry, 2008).

Depending on the type of meat, the gas composition varies (Table 2.1). Typically, fresh red meat is stored in MAP containing 80% O_2 and 20% CO_2, while cooked meats are usually stored under 70% N_2 and 30% CO_2 atmospheres (Walsh *et al.*, 2002; O'Grady and Kerry, 2008).

Table 2.1 Gas mixture recommended for modified atmosphere packaging of meat products. Modified from Ščetar *et al.* (2010).

Product	% Oxygen	% Carbon dioxide	% Nitrogen
Red meat	60-85	15-40	65-80
Cooked meat	-	30	70
Cooked ham in slices	-	40	60

As mentioned before, packaging affects the quality of stored meat products. The choice of films for packaging meat is largely determined by their moisture and gas permeability properties (Heinz and Hautzinger, 2007; Ščetar *et al.*, 2010). The most commonly-used materials for packaging are shown in Table 2.2. Most of the films used are moisture barriers in order to avoid excessive weight loss from the meat. Gas permeability is much more variable and is specific for individual polymers. For example, for retail cuts of fresh meat where the retention of bright red color is desired, packages with high oxygen transmission rates are used; whereas, for cuts of meat and processed products where storage life is the primary concern, packages with low gas transmission rates are used (Walsh *et al.*, 2002).

2.14. Microflora of various ready-to-eat meat products

Information about microorganisms implicated in the spoilage of ready-to-eat products is not as extensive as for raw meat products and cured meat products.

Table 2.2 Barrier properties of various plastic material commonly used in meat packaging. Modified from Walsh *et al.* (2002).

Material	O_2 transmission rate ($mL.m^2.day^{-1}$) (100% oxygen) 23°C, dry	Water vapour transmission rate ($g.m^{-2}.day^{-1}$) 38°C; 90% RH
Polyesters (PET)	50-100	20-30
High density polyethylene (HDPE)	2100	6-8
Polypropylene (PP)	3000	10
Polyamide (PA)	80	200
Polyvinylidene chloride (PVDC)	1.2-9.2	0.8-3.2
Polystyrene (PS)	2500-5000	110-160
Ethylene/vinyl acetate (EVAC)	12000	110-160

2.14.1. Fermented products

In the meat industry, lactic acid bacteria (LAB) and coagulase-negative cocci (CNC) are commonly used as starter cultures for fermented meat products. LAB are mainly responsible for the acidification that occurs within a product during the first days of fermentation, while CNC participate in the proteolytic and lipolytic processes that influences other sensory characteristics of the final product (Rantsiou *et al*., *2005).*

Tu *et al.* (2010) evaluated the microbial ecology of fermented ham, and identified the presence of diverse spectra of species and genera of microorganisms. However, two of the most dominant LAB known to play roles in meat fermentation, *Lactobacillus curvatus* and *Lactobacillus plantarum*, were not identified in their experiment. The microbial isolates which were identified by PCR-DGGE (polymerase chain reaction denaturing gradient gel electrophoresis) included *Lactococcus spp., Lactobacillus fuchuensis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *Lactis, Lactococcus garvieae, Lactobacillus sakei, Carnobacterium spp., Carnobacterium divergens, Staphylococcus saprophyticus, Staphylococcus arlettae, Staphylococcus xylosus, Microbacterium oxydans, Enterobacter agglomerans, Brochothrix thermosphacta, Leuconostoc citreum, Leuconostoc mesenteroides, Leuconostoc carnosum, Enterococcus faecium* and *Enterococcus faecalis.* Modern technology of fermented products generally apply or inoculate LAB in order to control this process, since these organisms

tend to be the sole agents of fermentation (Hammes *et al.*, 1990).

2.14.2. Cured hams

Dry-cured ham is produced in various regions of North America and Europe; yet, there are major differences between the production system in Europe and North America resulting in distinctive quality differences between their respective products (Ockerman, 2002). With respect to microbial quality, there is a lack of information regarding the spoilage process and most of the information available has characterized the microbial population of European hams.

In a study conducted by Martin *et al*. (2007), the microorganisms implicated in the spoilage process of Iberian dry-cured hams were characterized and it was found that the most predominant microbial groups were *Enterobacteriaceae* (*Serratia liquefaciens, Hafnia alvei and Enterobacter aerogenes*) which had a 33.3 % prevalence, and the Gram-positive, catalase-positive cocci *Staphylococcus lentus* and *Staphylococcus xylosus,* which made up a 22.2% frequency of total isolates. However, the authors also reported that lactic acid bacteria occurred at lower levels (11.1% of total isolates). Other species that have been isolated from cooked cured products include *Weissella viridescens, Carnobacterium divergens, C. piscicola, B. thermosphacta, Pseudomonas fragi, Ps. fluorescens* and *Ps. lundensis* (Metaxopoulos *et al*., 2002).

2.14.3. Cooked meat

Lactic acid bacteria are considered a major component of the microbial population found on various types of vacuum-packaged cooked sausages. *Lactobacillus sake* and *Lactobacillus curvatus* have been shown to be common species in these products (Korkeala and Björkroth, 1997). Hu *et al.* (2009) analyzed the predominant bacteria in sliced cooked ham made with pork formulated with sodium chloride, pentasodium tripolyphosphate, sodium ascorbate, sodium glutamate, sucrose, flavouring additives, soya isolate protein, potato starch, nitrite and water. In this study, the presence of *Leuc. mesenteroides, Chryseobacterium proteolyticum, Lactobacillus sake, L. curvatus,* uncultured *Leuconostoc sp., Brochothrix* sp., *Streptococcus thermophilus* and *Arthrobacter* sp., were detected (Hu *et al*., 2009). In a different study conducted by Hamasaki *et al.* (2003) it was found that the lactic acid bacteria implicated in the spoilage of commercially-available cooked meat (vacuum-packaged sliced loin ham and MAP wiener sausage) consisted on *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Lactococcus lactis* subsp. *lactis* and *Leuconostoc citreum*.

2.15. Characterization of microbial communities

Culture-dependent techniques have traditionally been used to identify and enumerate the different microorganisms that can be found in food products, and to enable the physical and metabolic characterization of isolated colonies (or clones) necessary for detailed examinations. However, culture-based methods are often limiting since selective media generally fail to mimic the conditions that a particular microorganism may need for proliferation in their natural habitat. Culture-based methods are further limited by the fact that many microbial phenotypic characteristics are shared across different species and genera, complicating the interpretation of data.

Thought to overcome potential biases arising from culture-based methods, molecular techniques have seen rapid acceptance for determination of the microbial biodiversity of various food products (Rantsiou and Cocolin, 2006), due to their high sensitivity and specificity that provide phylogenetic information based on comparative sequence analyses. The following sections review relevant culture-based and molecular methods for the analysis of food borne microorganisms.

2.15.1. Culture-dependent methods

Culture-dependent methods are based on growth of the bacterial species on synthetic media that resemble the conditions of the system from which the microorganisms are isolated. For the identification of lactic acid bacteria (LAB), the Man, Rogosa, Sharpe (MRS) medium is most commonly used (Alves *et al.*, 2006; Silvestri *et al.*, 2007). To provide a microaerophilic environment and enhance the growth of these microorganisms, the 'double layer' technique has been used to facilitate the growth of LAB (Rantsiou and Cocolin, 2006), after which the medium is kept at 45 – 50°C. For detection of CNC, Mannitol Salt Agar (MSA) medium is generally used due to its selectivity towards halotolerant species, with incubation at 30ºC for 48 h (Rantsiou and Cocolin, 2006; Gounadaki *et al*., 2008). Total viable counts may be obtained from Plate Count Agar (PCA) incubated at 30ºC for 48 h; *Enterobacteriaceae* from Violet Red Bile Glucose Agar (VRBGA) incubated for 24 h at 37°C (Gounadaki *et al*., 2008), and yeast and molds from Potato Dextrose Agar (PDA) incubated at 25ºC.

The API identification system (BioMerieux Inc., Marcy-l'Etoile, France) is often used for microbial identification (Rantsiou and Cocolin, 2006). For Gram-negative identification, the API

20E kit identifies *Enterobacteriacae* and group/species of non-fermenting Gram-negative rods; the API 20NE identifies Gram-negative non-*Enterobacteriaceae*, and the API Rapid 20E enables identification of Enterobacteriaceae.

Presently, the API/ID 32 range allows the identification of over 600 different species and it includes 15 identification systems covering all groups of bacteria such as *Listeria*, *Staphylococci*, *Enterobacteriaceae* and non-fermenting Gram-negative rods such as *Bacilli* and *Lactobacilli.*

The Biolog system (Biolog Inc., Hayward, CA, USA), on the other hand, is an identification/characterization system that can rapidly identify over 1,900 species of aerobic and anaerobic bacteria, yeast, and fungi. Options include a fully-automated system (OmniLog ID), a semi-automated system (MicroLog MicroStation) and manual-read systems (MicroLog 1 and 2). These products are based on the exchange of electrons produced during an organism's respiration based on utilization of different sole carbon sources, leading to a subsequent tetrazolium redox dye-based color change. Each of the 96 wells of the microtiter-style plate contains tetrazolium dye and a different sole carbon source; the tetrazolium dye changes from colorless (the salt form of the dye) to purple (the formazan crystal form of the dye) as actively-growing cells oxidize the different carbon sources (Garland and Mills, 1991; O'Hara, 2005).

In addition, there are also specialized media that allow the growth of certain microorganisms (selective media) and/or display a color change that provides biochemical information (differential media). For example, Baird Parker Agar and Vogel and Johnson Agar are selective and differential media commonly used in the detection of coagulase-positive *Staphylococcus aureus*. EMB Agar allows the detection and isolation of Gram-negative enteric bacteria based on lactose fermentation. MacConkey Agar, a selective medium for enteric Gram-negative bacilli, differentiates lactose fermentative from non-lactose fermentative microorganisms (Atlas, 1995).

Culture-dependent techniques have been the method of choice in microbiology for a long time. However, these approaches do not provide comprehensive information on the composition of microbial communities or information about the predominant bacteria in nature due to the difficulty in cultivating many microorganisms with stringent growth requirements. The implementation of new gene-based strategies to detect, identify and monitor bacteria in food products has made rapid advances over the past several decades.

2.15.2. Culture-independent methods

Culture-independent techniques have been developed, in part, to overcome the limitations of the classical culture-dependent approaches. The study of microbial diversity can now be achieved by using high-throughput sequencing approaches after direct nucleic acid extraction from mixed microbial populations without the need for their cultivation and isolation on agar medium (Rantsiou and Cocolin, 2006; Ercolini, 2013).

The desire for increased sequencing throughput has been accomplished with a variety of next generation sequencing techniques, such as 454 Pyrosequencing and Ion Torrent. The 454 Pyrosequencing method was the first technology to sequence and assemble entire bacterial genomes and the first non-Sanger technology to sequence an individual human genome (Rothberg and Leamon, 2008). The Pyrosequencing method evolved from individual reactions conducted within microliter scale volumes in 96- or 384-well plates, to the simultaneous sequencing of several hundred thousand picoliter scale reactions (Leamon and Rothberg, 2007). Traditional Pyrosequencing is based on a reaction that contains a pool of identical DNA templates and primers that anneal to known sites on each strand, to which a DNA polymerase is subsequently bound. Nucleotides are added individually to the reaction in series and repeated cyclically. The sequence information is based on the release of inorganic pyrophosphate (PPi) with every nucleotide incorporation. This PPi release initiates an ATP sulfurylase/luciferase enzyme cascade, converting the first PPi to ATP in the presence of d-luciferin and adenosine phosphosulfate (APS). Then, ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin, generating visible light in amounts that are proportional to the amount of ATP (Ramon *et al.*, 2003; Leamon and Rothberg, 2007, 2008). The total amount of signal (light) generated is captured quantitatively by a charge coupled device (CCD) camera and displayed as a peak in a pyrogram™. Each peak height is proportional to the number of specific nucleotides (A's, T's, G's or C's) incorporated during the nucleotide extension phase of the sequencing cycle (Ramon *et al*., 2003; Leamon and Rothberg, 2007, 2008).

Sample preparation involves fractionation of the DNA template into small fragments through nebulization. Adaptors are ligated to the DNA fragments, immobilizing the DNA template on 28 μm streptavidin-coated magnetic beads. The beads are emulsified in a PCR reaction mixture in oil emulsion (emulsion PCR), and the individual templates are amplified

several million-fold via PCR. The amplified template is retained on the DNA capture bead by extension along reverse primers covalently bound to the surface of the bead. Following amplification, the emulsion is broken. DNA is denatured and beads carrying single DNA templates are enriched and deposited into wells along a fiber optic slide. Smaller beads carrying immobilized enzyme are also deposited into each well (Leamon and Rothberg, 2008).

Ion Torrent technology functions in a similar fashion to 454 Pyrosequencing. Sequencing is performed by sequentially flowing nucleotide bases into the PCR reaction one by one, measuring their incorporation by the molecular changes that occur. However, unlike pyrosequencing (where light is released and a chemiluminescent reaction is measured), the Ion Torrent system directly measures the release of H^+ (protons) from the growing DNA strand. This is accomplished through the use of millions of nano-sized wells etched into a semiconductor chip that each individually function as a pH meter, directly measuring the hydrogen released during the polymerization of DNA. The H^+ concentration creates a positive voltage near the gate region of the transistor, which then results in a proportional change in the current flowing through the transistor (Merriman and Rothberg, 2012) that can be correlated to the specific number of bases that were added to the growing ds-DNA chain.

Preparation and amplification steps are more or less similar (emulsion PCR) to that used during 454 Pyrosequencing, with beads containing amplified DNA being loaded into individually-etched nano-wells. The chip is then placed in the sequencing instrument and the sequencing process start (Merriman and Rothberg, 2012).

Overall, these molecular techniques are commonly used in environmental microbiology. However, their implementation to microbiological studies in the food sector are still in its early stages, giving a promising future for understanding the microbial changes that occur in food products under different conditions. Over the last few years, some analyses targeting 16S rRNA have been used to study the microbial biodiversity of cooked ham (Vasilopoulos *et al*., 2008; Hu *et al*., 2009), lactic acid bacteria on sliced cooked ham, turkey and chicken (Audenaert *et al.*, 2010).

However, the application of DGGE has some limitations. First, identification of microorganisms by PCR-DGGE (e.g., detection of a specific band on the DGGE gel) can be achieved only if the concentration of particular microorganisms in the test system is more than 10^4 CFU g^{-1}. Lower concentrations of cells generally cannot be reliably detected due to the weak

intensity of the resultant bands. Secondly, the presence of multiple copies of the ribosomal genes may result in several band patterns per species in high-resolution PCR-DGGE analysis (Rantsiou and Cocolin, 2006). To overcome this issue, some authors have proposed the use of the *rpo*B gene that encodes for the beta subunit of the RNA polymerase gene and which usually exists in a single copy (Rantsiou *et al.,* 2005).

2.16. Bacteriocins

Bacteriocins are ribosomally-synthesized peptides with antimicrobial activity against other bacteria (Gálvez *et al.,* 2007; Nishie *et al.,* 2012). Bacteriocins produced by LAB have been the most intensively studied due of their GRAS (generally recognized as safe) status, as designed by the U.S Food and Drug Administration (FDA), indicating they do not have a toxic effect on eukaryotic cells and are safe to use as food preservatives or for pharmaceuticals purposes (Gálvez *et al.,* 2007; Nishie *et al.,* 2012). Also, bacteriocins are suitable for industry use because they become inactivated by digestive proteases and therefore tend to have little influence on the gut microbiota, they are usually pH- and heat-tolerant, and their genetic determinants are usually encoded extrachromosomally (on plasmids) thereby allowing their manipulation (Gálvez *et al.,* 2007).

2.16.1. Bacteriocin classification and mode of action

Bacteriocins are generally subdivided into various classes according their mode of action, molecular size and physical properties. Currently, they are classified as class I (modified peptides, lantibiotics), class II (unmodified peptides, non-lanthionine containing), and class III (large proteins, heat unstable) (Güllüce *et al.,* 2013).

Class I bacteriocins are known as lantibiotics and they are small, heat-stable peptides with low molecular weight (<5 kDa) (Balciunas *et al.,* 2013; Güllüce *et al.,* 2013). Bacteriocins belonging to this class go through a post-translational modification resulting in the formation of the unusual amino acid, lanthionine (Lan), and β-methyl-lanthionine methyllanthionine (MeLan). Nisin is the best known example from this group (Deegan *et al.,* 2006; Martin-Visscher *et al.,* 2008; Güllüce *et al.,* 2013). Lantibiotics are further divided into type A and B. Class Ia lantibiotics are linear peptides that inhibit bacteria by pore formation in the bacterial membrane. In contrast, Class Ib lantibiotics are more rigid, and have a globular structure (Deegan *et al.,* 2006; Nishie *et al.,* 2012; Güllüce *et al.,* 2013).

Class II bacteriocins are the largest group and comprised of small thermostable peptides (<10 kDa) that do not undergo post-translational modifications. Bacteriocins from this class have an amphiphilic helical structure allowing their insertion into the cytoplasmic membrane of the target cell causing membrane depolarization and cell death (Balciunas *et al.*, 2013; Güllüce *et al.*, 2013). This group is divided into three subclasses IIa (pediocin-like), IIb (lactocin G) and IIc (lactocin B), according to Balciunas *et al.* (2013).

Subclass IIa bacteriocins are small (37 to 48 amino acid residues) and are know to be very specific against *L. monocytogenes.* They have in common the amino acid consensus sequence YGNGVXC at their N terminus and a conserved disulfide bridge (Nishie *et al.*, 2012; Balciunas *et al.* 2013). Subclass IIb includes heterodimeric bacteriocins, meaning that two peptides must be combined together in order to be functional (Gülüce *et al.*, 2013). Finally, subclass IIc is comprised of bacteriocins that have a covalent bond between C and N terminals, resulting in a cyclic structure (Nishie *et al.*, 2012; Balciunas *et al.*, 2013; Gülüce *et al.*, 2013). Relatively few of these bacteriocins have been identified, but it is known that carnocyclin A, which is produced by *Carnobacterium maltaromaticum* UAL307, forms anion-selective pores in its target organisms, while enterocin AS-48 forms non-selective pores due to the dissipation of potassium ions as well as other small solutes out of the cell (Gong *et al.*, 2009).

2.16.2. Mechanisms of resistance to bacteriocins

Several factors have been reported to contribute to the development of resistance in bacteria towards a bacteriocin. Mechanisms contributing to class IIa bacteriocin resistance are the most-studied due to the fact most of the bacteriocins reported so far belong to this class.

The sigma 54-dependent operon, *mpt*ACD, has been described to be involved in the sensitivity to antibacterial peptides (Ramnath, 2004; Severinov *et al.*, 2011). The operon *mpt*ACD encodes the A, B, C and D subunits of the PTS permease of the mannose family, which is the major sugar uptake system in *Firmicutes* and *Gammaproteobacteria* (Kjos *et al.*, 2010).

2.17. Biopreservation

Biopreservation can be defined as a natural preservation method to improve the safety and stability of food products by using specific microorganisms and/or their metabolites without negatively affecting the sensory quality of the food product. Due to an increase in consumers' concern about the use of chemical additives and antibiotics to ensure the safety of processed

foods, efforts have been made to introduce biological preservatives (Gálvez *et al.*, 2007). Different types of packaging techniques have been used in combination with different storage techniques in order to extend the shelf-life of meat.

For more than two decades, *L. monocytogenes* has presented food safety challenges, especially in ready-to-eat products, where the risk of listeriosis is higher because the product typically becomes contaminated after processing (Alves *et al*., 2006). An effective strategy of biopreservation for inhibiting this microorganism is important due to its ability to survive under a broad range of physicochemical conditions (e.g., from -0.1 to 45ºC, low pH (3.5) and salt content as high as 10% (Duché *et al.*, 2002).

Among the wide array of strategies currently used for food preservation, antimicrobial products such as bacteriocins, which are defined as antimicrobial proteins with bactericidal activity towards closely related species (Quintavalla *et al.*, 2002), combined with novel technologies such as active packaging, have received more attention due to their great potential for extending storage life and safety.

So far, there are two bacteriocins licensed as food preservatives by the FDA. Nisin, which is produced by *Lactococcus lactis* subsp. *lactis*, is approved for use in over 40 countries and has been used as a food preservative for over 50 years (Cleveland *et al.*, 2001), and Micocin®, a biopreservative containing the three pasteurized bacteriocins produced by C*arnobacterium maltaromaticum*, which has been tested and recently approved (2010) in several countries such as Canada, Colombia, Costa Rica, Mexico and the United States. However, in meat products, Nisin has not been very successful as a preservation system, because of its low solubility at the pH of meat, uneven distribution, binding to meat proteins, and lack of stability since its activity has been observed to decrease rapidly with time, especially at room temperature (Chung *et al.*, 1989). Micocin® may offer a better industrial alternative for the control of *L. monocytogenes* outbreaks and food product recalls. Its advantage relies on its resistance to proteolysis and stability at different temperatures and pH values (Martin-Visscher *et al*., 2008), maintaining control of pathogens over extended periods of time.

Some authors have reported the inhibition of *L. monocytogenes* by adding bacteriocin-producing GRAS cultures into the food. For example, *Lactobacillus sakei* has been studied on cooked ham (Alves *et al.*, 2006) and also on Brazilian sausages (De Martinis and Freitas, 2003). Pediocin PA-1, which has a broad inhibitory spectrum, has been successfully-applied in dry-

fermented sausages and cooked cured frankfurters for controlling the growth of *L. monocytogenes* and *Clostridium perfringens* (Nieto-Lozano *et al.*, 2010).

3. INVESTIGATION OF LOW-SODIUM SLICED COOKED BOLOGNA QUALITY USING CULTURE-DEPENDENT AND CULTURE-INDEPENDENT TECHNIQUES

3.1. Abstract

Culture-dependent and culture-independent-techniques were used to analyze sliced cooked bologna formulated with 1, 2 and 3% (w/w) salt and surface-inoculated with *Brochothrix thermosphacta*, *Pseudomonas fluorescens* and lactic acid bacteria (i.e. *Leuconostoc mesenteroides* and *Lactobacillus curvatus*). The meat product was then stored at 4°C under aerobic or vacuum-packaged conditions. Extraction of bacterial DNA from bologna slices at day 15, followed by Ion Torrent 16s rRNA sequence analysis, was performed in order to characterize how bacterial diversity responded to the different treatments.

In general, plate counts using selective media showed that vacuum packaging slowed bacteria growth compared to growth on bologna stored aerobically. However, there was no significant effect ($p>0.05$) of 1 and 2% (w/w) salt levels on microbial growth; whereas, the 3% (w/w) salt level significantly increased lag phase. Ion Torrent sequence analysis demonstrated that the predominant bacterial spoilage group was lactic acid bacteria but also confirmed the presence of other indigenous microbial genera, such as *Staphylococcus*, *Serratia* and *Salmonella*.

Under the conditions employed during this study, it was found that a decrease of pH, either in aerobic or vacuum-packaged treatments, was most probably due to the dominance of LAB. On the other hand, with respect to water activity it was found it was significantly influenced ($p<0.05$) by sodium concentration. An increase in numbers of spoilage bacteria was consistent with a drop in redox potential values, measured *in situ*, which also might indicate an asymmetric distribution of organisms on the sliced cooked bologna.

Overall, decreasing the salt concentration from 2% to 1% did not have a significant effect on bacterial growth in this meat system. The utilization of Ion Torrent high-throughput sequencing in this study allowed an enhanced description of the total microbial community present, and in particular, the diversity of lactic acid bacteria.

3.2. Introduction

Sodium is a critical element that has an important role in body physiology, regulating extracellular fluid as well as the active transport of molecules across cell membranes (Doyle and Glass, 2010). However, excessive dietary intake of sodium has been associated with the development of hypertension, cardiovascular disease and, possibly, other health issues (Matthews and Strong, 2005). In Canada, statistics have reported that one in four Canadians is living with high blood pressure. A survey conducted in 2004 revealed that all age groups (from 1 year to 70 years of age) consumed more that 3,100 mg of sodium, or 7.9 g of salt (NaCl), a day (CTAC, 2009). The Canadian food industry, since 2007, has been challenged with implementing the sodium reduction strategy proposed by the Food Standards Agency (FSA), with aims to be within the targeted acceptable limits (less than 2,300 mg per day and less than 1,500 mg per day for those that are 50 and older) by 2020 (CTAC, 2009; Stringer and Pin, 2005; Matthews and Strong, 2005). However, in October 2013, Hypertension Canada proposed to increase the recommended amount to 2,000 mg of salt per day since a sodium reduction to 1,500 mg per day has been considered not feasible and scientific research has also demonstrated that a sodium reduction to 2,000 mg significantly improves blood pressure.

In North America, the meat industry is considered to be the second greatest contributor of dietary sodium after cereal products (Matthews and Strong, 2005). In meat products, sodium chloride is one of the most frequently used ingredients because of its many beneficial functions (Matthews and Strong, 2005). It plays an important role in flavour and texture, as well as a crucial role in the microbiological safety of meat products. As a preservative, sodium chloride reduces the water activity, controlling growth of meat spoilage bacteria and food pathogens. For instance, a reduction in salt levels may reduce product shelf-life and also compromise food safety (Ruusunen and Puolanne, 2005; Stringer and Pin, 2005; Doyle and Glass, 2010).

Determining how meat product safety may be compromised by lowered sodium content is of obvious importance. Studies have demonstrated how different groups of microorganisms behave in broth culture under certain pH, water activity and temperature under the influence of different sodium chloride concentrations. However, information obtained in broth culture offers limited information, due to the fact bacteria growth is affected by the food matrix, as well as by indigenous food microorganisms.

To date, several studies have reported the different approaches industry has taken in order to replace sodium chloride and the effects of reduced salt levels have had on the sensory quality and functional properties of meat products (Sofos, 1983; Ruusunen *et al.*, 2005; Doyle and Glass, 2010), but there is a lack of information on microbial behavior and food safety on these types of products.

In this study, the effect of three different sodium chloride concentrations (1, 2, and 3% w/w) on microbial growth was investigated using culture-dependent and culture-independent techniques. The postulate of this study is that due to the importance of sodium chloride on water holding capacity, any reduction of salt will increase the water activity (a_w) of the product, thereby affecting microbial growth. Thus, it is hypothesized that a reduction on sodium chloride levels will increase microbial growth, resulting in increased acidification of the product as well as a salt concentration-dependent reduction in bologna microbial quality.

3.3. Material and Methods

3.3.1. Experimental design

The aim of this first study was to examine the effect of salt concentration on the proliferation of indigenous and introduced microflora in a model cooked meat (bologna) system. Sliced bologna was manufactured in the Meat Pilot Plant (4°C) located in the Department of Food and Bioproduct Sciences at the College of Agriculture and Bioresources, University of Saskatchewan, and was formulated with 1, 2 and 3% NaCl. The bologna was produced in large batch quantities, sliced and finally stored at 1°C for subsequent handling.

Sliced samples of each bologna formulation were surface-inoculated with 0.1 mL of a defined cocktail of spoilage microorganisms (described in detail below), including *Pseudomonas fluorescens* and *Brochothrix thermosphacta* and the lactic acid bacteria (LAB) *Lactobacillus curvatus* and *Leuconostoc mesenteroides.* As a control, packages containing 5 slices were inoculated with sterile distilled water and packages containing 5 slices of inoculated bologna were incubated under aerobic or vacuum-packaged atmospheric conditions. As the product was not sterile, indigenous organisms were also present and expected. One preliminary study was conducted at 8°C and two subsequent studies were carried out at 4°C. The change in numbers of the different microorganisms was first monitored every third day over a 40-day incubation period during the first trial, every third day over 30 days during the second trial (Fig 3.1).

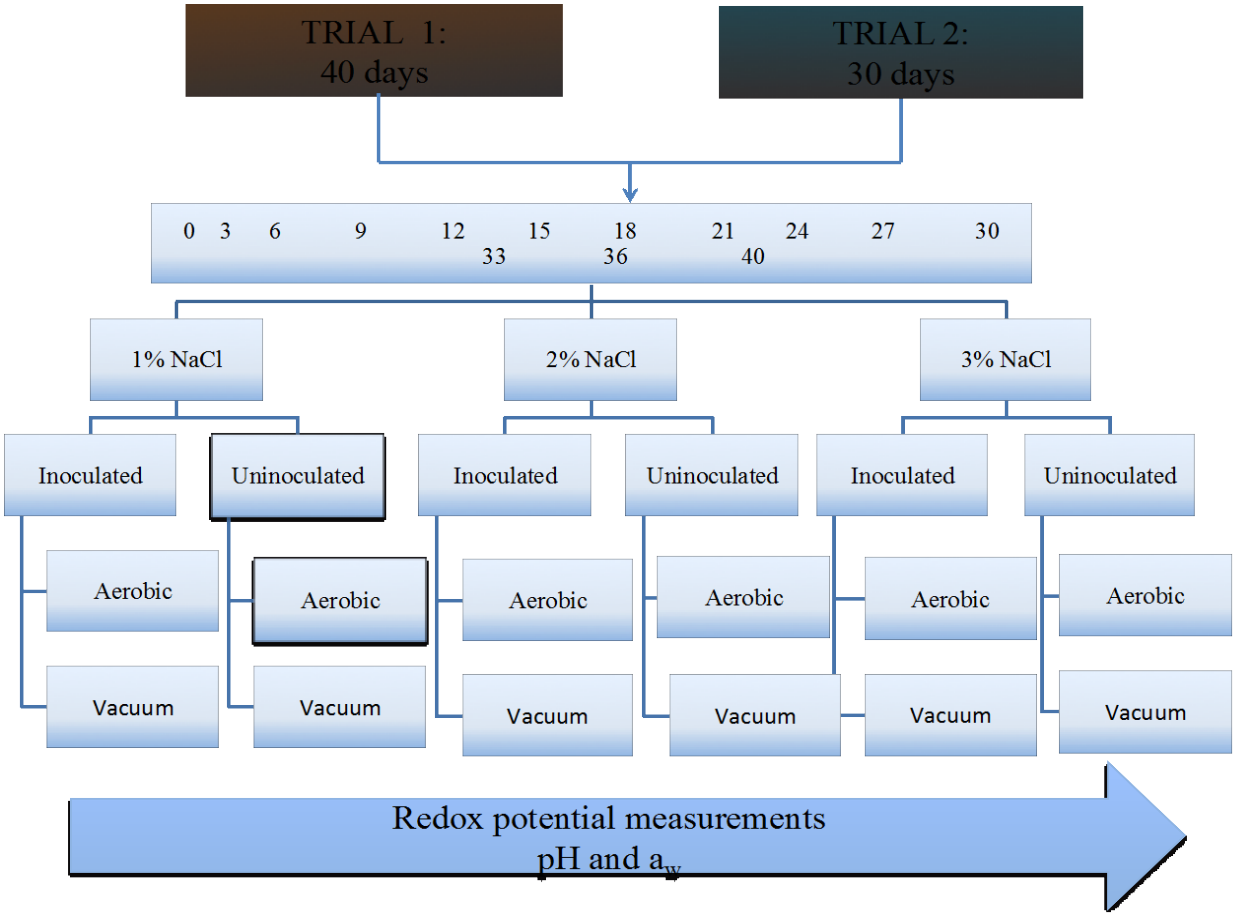


Figure 3.1 Schematic diagram illustrating the experimental procedure conducted over 40 days during Trial 1 and over 30 days during Trial 2 at a temperature of 4°C. Each trial utilized cooked bologna formulated with 3 different (1, 2 and 3% NaCl) sodium concentrations, which were then stored under aerobic or vacuum-packaged conditions. Each treatment included uninoculated control samples as well as bologna samples inoculated with spoilage bacteria. *In situ* bologna redox measurements (mV) were collected continuously over the storage period, whereas pH and a_w measurements were made at intervals indicated in the diagram timeline.

Community dynamics (determined as the change in the number of microbes) of predominant spoilage bacteria were monitored during refrigerated storage temperatures as mentioned above using traditional microbiological methods based on plate counts on selective media, as well as by molecular techniques (by determining the community biodiversity and sequence abundance) where DNA was extracted and a partial 16S rRNA gene (the V5 region) was amplified for sequence analysis using Ion Torrent sequencing.

Over the course of this research, one biological replicate (Trial 2) was carried out after completion of Trial 1. However, considerable variation was observed between the two trials regarding the variability in the growth and abundance of *Pseudomonas fluorescens,* which was greater than would be expected if random processes were the sole contributing factor despite the fact that the same concentration of *P. fluorescens* was inoculated on the bologna samples. Consequently, the data for this microorganism was not averaged over the two trials (Fig 3.2). Based on the fact that biological replicates were processed on different days, some variation may be attributed to differences between the indigenous microflora between lots, meat chemistry, meat handling process, and maintenance/condition of the processing area.

3.3.2. Meat handling

Fresh pork meat (5 d post-mortem) was obtained from a commercial meat processor through a local supplier. The meat (handled in approximately 10 kg batches) was cut, minced through a 6.5 mm hole plate (Biro Grinder, Marblehead, OH, USA, model AMFG-24) and transferred into 30 lb-capacity vacuum bags (mylar/polyethylene vacuum pouch, 3 mm thick, oxygen permeability of 7.7 $mL/m^2/24$ hr), vacuum-packaged (-0.9 bar, Roshermatic Type VM-20, Osnabruck, Germany) and kept at 1°C until further processing.

3.3.3. Manufacture of cooked bologna

Pork meat and fat were first reground through a stainless steel plate (3/16”) using a 3.9 mm hole grinder and then kept separate from each other. Each formulation targeted a fat content of 20.0% and a protein content of 11.0%. The level of meat, backfat, starch, Prague powder, sodium tripolyphosphate, sodium erythorbate and German Wiener Seasoning were kept constant for all treatments. It is important to mention that bologna production was randomized and that some ingredients used in this bologna formulation contained a certain quantity of sodium and thus the amount of sodium provided by each ingredient was taken into account. The level of water and salt varied according to the sodium concentration treatment, as shown in Table 3.1.

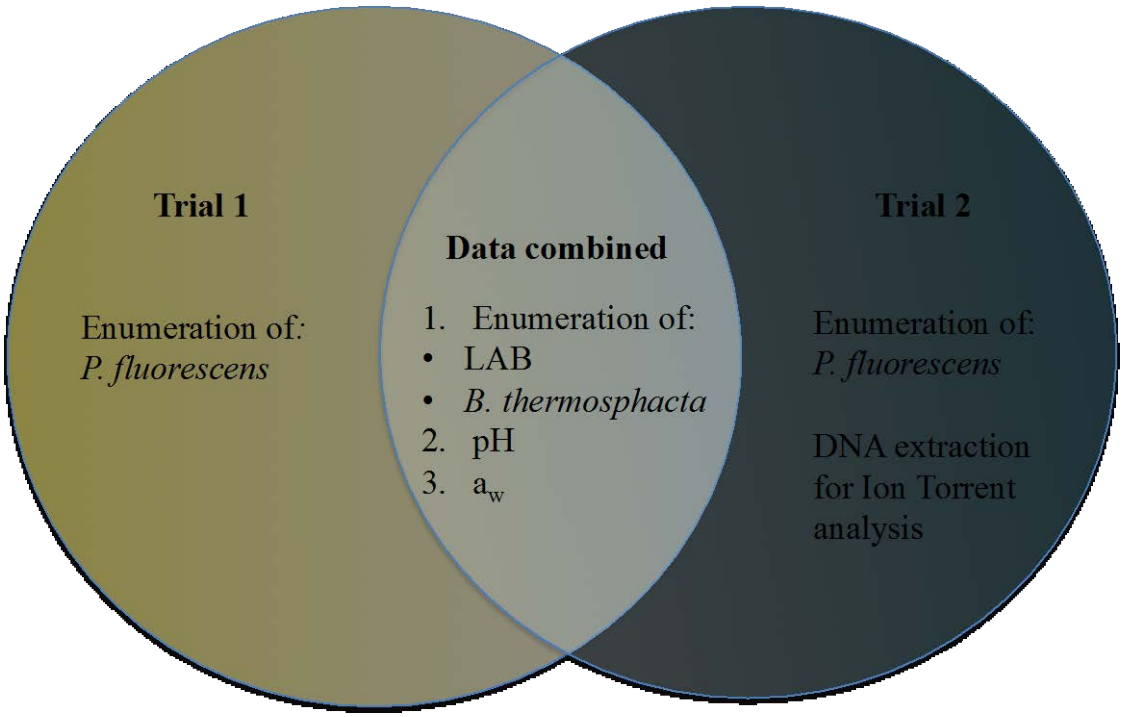


Figure 3.2 Diagram illustrating the analysis of data from trials 1 and 2. The overlapping area indicates the data obtained throughout 30 days that was averaged. Ion Torrent analyses were conducted from samples obtained in Trial 2.

Table 3.1 Bologna formulations (%, w/w) incorporating three different levels of NaCl

Ingredients	1%	2%	3%
Pork leg muscle, %	59	59	59
Pork backfat, %	15	15	15
Ice water, %	21.9	20.9	19.9
NaCl, %	0.7	1.7	2.7
Prague powder, %	0.3	0.3	0.3
Sodium erythorbate, %	0.05	0.05	0.05
Sodium tripolyphosphate, %	0.4	0.4	0.4
German wiener seasoning, %	0.6	0.6	0.6
Wheat flour, %	2	2	2

* Prague powder contains 6.25% sodium nitrite and 93.75% salt.

First, the ground meat was mixed with the seasoning ingredients and half of the ice water in a large-bowl chopper (Hobart, Troy, OH, USA, model #84181D) for 1 min with a bowl speed setting of 2 and a knife speed of 4. Afterward, pork backfat and the remaining water were added and chopped for 2 more min, reaching a temperature of 6°C by the end point. Meat was then transferred and run twice through an emulsification mill (Type 1E-75F, Alexanderwerk, Remscheid, DE) with a final temperature of no greater than 16°C. The meat batter was then transferred to a vacuum tumbler (Model VSM-150H, Glass, Frankfurt, DE) and in order to release the air trapped in the meat batter matrix, the meat batter was vacuum-pulled (Model VSM-150H, Glass, Frankfurt, DE), and tumbled for 3 min twice. Approximately 1 kg of tumbled meat was then stuffed using a hydraulic stuffer (Mianca Equipamientos Carnicos, model EL-20SL, Barcelona, ES) into 63 mm diameter plastic casing (Walsroder KFS MATT red, Art-No.: 40216663, CaseTech GmbH and Co., Walsrode, DE) and sealed with aluminum clips.

The resultant bologna chubs were then cooked for 2 hr by immersion in a water bath (~200 L). The cooking cycle consisted of 4 stages: 1) 30 min at 50°C, 2) 30 min at 60°C, 3) 30 min at 70°C, and 4) 30 min at 75°C. The final internal temperature of the product was 71°C. Internal temperature was monitored throughout cooking using both a hand-held Fluke digital thermometer (Type T Fluke 51 II thermometer, Fluke Corp., Everett, WA, USA) with a chromel-alumel thermocouple probe and a 8 channel data logger (Model 692-0000 Barnant Scanning Thermocouple Thermometer, Barnant Co., Barrington, IL, USA) with three copper constantan thermocouples positioned in geometric center of the bologna chubs.

After cooking, samples were immediately cooled in an ice and water mixture for one hour and stored at 1°C until the next day. Then, three bologna chubs from each salt level were chosen randomly for proximate analyses; moisture, fat, protein and ash contents (Table 3.2) were determined using AOAC (1990) methods. The remaining product was then sliced into 3-4 mm thick slices using a disc cutter, vacuum-packaged and stored at 1°C until time of inoculation.

3.3.4. Bacteria and preparation of inocula

Based on a literature review, the selected bacterial strains used throughout this study included *Lactobacillus curvatus* (DSM 20019), *Leuconostoc mesenteroides, Pseudomonas fluorescens* and *Brochothrix thermosphacta* (Food isolates obtained from the Microbiology Laboratory Culture Collection at University of Saskatchewan). Frozen stock cultures were grown over night after which 1 mL of the culture was used to inoculate a fresh flask containing the

appropriate broth media. Lactic acid bacteria were grown in 100 mL Man-Rogosa-Sharpe (MRS) (Becton Dickinson, Franklin Lakes, NJ, USA) at 30ºC for 24 hr; whereas, *Pseudomonas fluorescens* and *Brochothrix thermosphacta* were grown in 100 mL Brain Heart Infusion (BHI) (Becton Dickinson, Franklin Lakes, NJ, USA) broth at 25ºC. The cells were then harvested by centrifugation (10000 x g for 10 min), washed twice and resuspended in 0.1% peptone water. The four strains were then aseptically combined and diluted in peptone water to a concentration of ~10^3 - 10^4 CFU/mL for each organism.

Table 3.2 Proximate composition of cooked bologna formulated with three different levels of NaCl.

%NaCl	%Moisture	%Protein	%Fat	%Ash
1	64.15 ± 0.02	12.24 ± 0.00	20.79 ± 0.05	2.05 ± 0.01
2	63.52 ± 0.11	12.35 ± 0.09	20.22 ± 0.09	2.95 ± 0.03
3	62.52 ± 0.03	11.91 ± 0.97	20.24 ± 0.14	4.03 ± 0.05

[1]Protein was calculated as total nitrogen x 6.25

3.3.5. Inoculation procedure

Sliced bologna was kept for 24 hr at 4°C. After thawing, packages were aseptically open and slices were aseptically kept inside a biosafety cabinet. The inocula cocktail was then spread over the entire surface of each slice using 100 µL of the inoculum using sterile L-shaped glass rods; slices were then stored aerobically by simply twisting the bag or under vacuum packaging conditions (KOMET Vakuun-Verpacken, KOMET Maschinenfabrik GmbH, Plochingen) using bags (200 × 300 mm) of plastic barrier film (75 microns nylon/polyethylene, oxygen transmission rate 63 cc/sq. m / 24 hr at 23°C). Each package contained 5 slices. All samples were stored at 4°C for 30 days.

3.3.6. Microbial analyses

Bologna slices (10 g) were aseptically removed from the package and transferred to a sterile stomacher bag (Seward Laboratory System Inc., UK) containing 90 mL of sterile buffered peptone water, and then homogenized for 5 min at 230 rpm using a stomacher (LAB Blender 400, PBI, Milan, Italy). Appropriate serial dilutions of the stomached materials were then prepared using 0.1% sterile peptone water (Becton Dickinson, Franklin Lakes, NJ, USA). In

order to enumerate the microorganisms, 0.1 mL of each dilution was spread onto triplicate plates of the appropriate culture medium.

Lactic acid bacteria (LAB) viable counts were obtained on MRS medium incubated for 72 hr at 30ºC; *Pseudomonas fluorescens* counts were obtained by incubation on cephaloridine fucidin cetrimide (CFC-agar, Oxoid Ltd., Basingstoke, UK) agar at 25ºC for 48 hr and *Brochothrix thermosphacta* was enumerated on streptomycin thallous acetate actidione (STAA, Oxoid Ltd., Basingstoke, UK) agar incubated for 48 hr at 25ºC.

3.3.7. Turbidimetric assay

Generally, studies have screened the effects of salt concentration on the growth of microorganisms in combination with other factors, such as pH, temperature and/or other type of salts (Durack *et al.*, 2013). However, there is scare information on the microbial response within these salt ranges having only sodium concentration as a variable of study.

In this study, the individual growth responses of *P. fluorescens*, lactic acid bacteria, *B. thermosphacta* and *L. monocytogenes* were evaluated over a 1-3% NaCl concentrations range by measuring culture optical density at 600 nm over time. For this purpose, broth culture media (see below) was adjusted to a final concentration of 1, 2 and 3% NaCl. *Pseudomonas fluorescens*, *B. thermosphacta* and *L. monocytogenes* were grown overnight on BHI broth (Becton Dickinson, Franklin Lakes, NJ, USA) and LAB were grown on MRS (Becton Dickinson, Franklin Lakes, NJ, USA), and used to inoculate flask cultures containing the different media. The inocula concentration used was in the range of 10^2 to 10^3 CFU/mL. Inoculated flasks were then immediately placed in shaking (200 rpm) water bath maintained at 15°C. Growth determinations for each treatment were performed in triplicate and after data collection, the slope of the natural log-transformed (Ln) OD measurements vs time (hr) were plotted in order to obtain specific growth rates (μ_{max}). Finally, these data were analyzed as fixed effect model using the Proc Mixed Procedure of SAS (SAS, Inst. Inc., Cary, NC).

3.3.8. Water activity (a_w)

Water activity was measured with a calibrated hygrometer (AquaLab, Rotronic, Bassersdorf, Switzerland) following the manufacture's recommendations. Triplicate measurements were made per treatment.

3.3.9. pH measurements.

The pH was measured by immersing the pH electrode in the stomacher bag containing the homogenized sample, prepared as indicated in section 3.3.5. The pH meter (Fischer Scientific, Accumet AB15 plus Brinkmann Instrument Canada, Nepean, ON) was calibrated prior to taking pH measurements. Measurements were conducted in triplicate.

3.3.10. Redox potential monitoring

Measurement of sliced bologna redox was performed using microelectrodes connected to a data logger (51x Micrologger, Campbell Scientific Inc., Edmonton, AB). The redox potentials (E_h) of sliced bologna were continuously recorded over time. Two microelectrodes and one 2 mm reference electrode (World Precision Instruments Inc., FL, USA) were inserted into each bologna package, with 1 microelectrode located on the bologna slice surface and other microelectrode positioned between bologna slices. The E_h value was calculated from the equation (equation 1):

$$E_h = E_{meas.} + E_{ref.} \qquad \text{Equation 1}$$

Where E_{ref} is the potential of the calomel electrode and E_{meas} is the measured potential of the platinum electrode in Volts (Kukec *et al*., 2002).

In order to create and maintain the proper packaging conditions, acoustic sealant (Code # 93170X 330, Tremco, Canada) was used to seal the top of the bag before the package was vacuum packaged. The sealant also served to reduce “dead” spaces between the bologna product and the electrodes.

Redox measurements were conducted for Trial 1 and 2. However, due to the variability of values obtained during Trial 2, only E_h values obtain during Trial 1 were taken into account.

3.3.11. DNA extraction and PCR amplification

Based on the results from the plating work, culture-independent analyses were only performed on DNA extracted from 1% and 3% NaCl treatments at day 15, due to the fact number of spoilage bacteria were high in both treatments and significantly different from each other. However, in this research study, only samples obtained from Trial 2 were analyzed.

In order to extract DNA directly from different cooked bologna treatments, 1 mL of the first 10-fold dilution prepared for microbial cultural analyses was used. These materials were

then subjected to DNA extraction using the InstaGene Matrix protocol (BioRad, Hercules, CA) according to the manufacturer's protocol.

3.3.12. Ion Torrent 16S rRNA gene sequencing

Sample preparation for Ion Torrent sequencing was performed according to Bondici *et al.* (2013), as follows. Ion Torrent sequencing and data processing were carried out using Torrent adapter A (forward) and adapter P1 (reverse), which were annealed to sequencing primers specific for the V5 region of the 16S rRNA gene. The forward primer included a key tag and a multiplex identifier (MID). The sequence of these primers were F5′-**CCATCTCATCCCTGCGTGTCTCCGAC**TCAGMIDGATTAGATA CCCTGGTAG and R5′-**CCTCTCTATGGGCAGTCGGT**-- **GAT**CCGTCAAT-TCCTTTRAGTTT, respectively, where the single underlined sequence represents the target region-specific primer, the double underlined sequence is the key tag, and sequences in bold are the adapters. The MIDs used for each bologna treatment were: AGCGT (Aerobic, 1% NaCl), TAGAG (aerobic, 3% NaCl), TCGTC (vacuum packaged, 1% NaCl), ACATA (vacuum packaged, 3% NaCl), ACGCG (uninoculated aerobic, 1% NaCl), AGACT (uninoculated vacuum packaged, 1% NaCl), CGTGT (uninoculated aerobic packaged, 3% NaCl) and CTCGC (uninoculated vacuum packaged, 3% NaCl). PCR reactions were carried out in 50 μl volumes containing 2 μl of template DNA, 1.0 μmol l^{-1} each primer, 1.0 U Taq polymerase (Invitrogen, Carlsbad, CA, USA), 1.0 μmol l^{-1} $MgCl_2$, 1.0 X PCR buffer (both provided with the Taq polymerase) and 4.0 μmol l^{-1} of deoxyribonucleotide triphosphates (dNTPs). Cycling conditions consisted of a 5-min denaturing step at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 57°C and 45 s at 72°C, and a final elongation step of 10 min at 72°C. The PCR amplicons were purified using a Qiagen purification kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions and quantified using a Nano-Drop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Libraries were sequenced using an Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA, USA) with a 314 chip at the Biotechnology Research Institute (Montreal, Quebec, Canada). Sequence data were processed using RDP pyrosequencing pipeline tools. The pipeline Initial Process Tool was used to trim and remove low-quality sequences, and sequences shorter than 100 bp. Quality sequences were submitted to the RDP classifier with a bootstrap cut-off value of 50%.

3.3.13. Statistical analysis

Water activity and pH data were analyzed using the fixed effect model of SAS (SAS, Inst. Inc., Cary, NC). The fixed effects on the model were salt concentration, storage condition, sampling day, salt concentration by condition, salt concentration by day, storage condition by day and salt concentration by storage condition by day and the random effect was replication.

The experiment was conducted twice at 4°C. Bacterial counts obtained on each selective media was subjected to analysis of variance (ANOVA) using the PROC GLM procedure in SAS version 9.3 software and comparison of treatments was determined with the Tukey test.

Microbiological data obtained over the first 30 days (Fig 3.2) was analyzed as repeated measures with a randomized design using the mixed procedure model of SAS. Approximation on degrees of freedom was used with the Kenward-Roger adjustment on standard errors. In both models, significance was declared at $p<0.05$ and the pdmix SAS macro was used to convert mean output to letter groupings (Saxton, 1998).

3.4. Results and Discussion

3.4.1. Optimization of the experiment results

3.4.1.1. Microbiological analysis

Microbial numbers were first followed during a preliminary study where conditions included a temperature of 8°C (slight temperature abuse) under aerobic and vacuum-packaged conditions for 12 days.

Figure 3.3 illustrates the growth of LAB, *B. thermosphacta* and *P. fluorescens* in cooked bologna formulated with 1, 2 and 3% NaCl. In general, it was possible to observe that salt did not affect spoilage bacteria growth since by day 8, bacterial counts were at or above 8 log CFU g^{-1}. As it was illustrated, when comparing the 2% and 3% NaCl bologna formulae, during the first 4 days of storage under aerobic conditions there was essentially no difference in growth between the different spoilage bacteria. Also in these two treatments, it was possible to observe that *P. fluorescens* and LAB counts reached ~8 log CFU g^{-1} after only 8 days of storage. On the other hand, on bologna formulated with 1% NaCl, more rapid growth of LAB and *B. thermosphacta* occurred over the first 4 days, with an apparent suppression of *P. fluorescens* growth over the same period. *Pseudomonas fluorescens* is an obligate aerobe, and thus it was expected this microorganism would be dominant during the early spoilage process. It should be noted,

however, that the initial inoculum size of this bacteria was approximately 1 log CFU g^{-1} lower than the LAB and *B. thermosphacta* inocula, a difference that may account for the early domination of these microorganisms during the spoilage process.

When the effects of vacuum packaging (Figure 3.3B) were compared to aerobic packaging, an increase in the lag phase of microorganisms, independent of sodium concentration, was observed. However, by the end of the trial period, LAB counts increased to over 9 log CFU g^{-1} in bologna formulated with all salt concentrations. On the other hand, a decline in numbers of viable cells to near to the minimum detection limit was determined for *P. fluorescens* cells by day 12 in all treatments; the variability of ecological determinants such as reduction of oxygen by vacuum packaging, reduction in the availability of nutrients due to the increase of bacteria and the presence of end products such lactic acid produced by LAB, may have strongly affected the growth of this spoilage microorganism.

3.4.1.2. Chemical analysis

Chemical analysis showed that water activity (a_w) remained constant (0.97-0.98) over the duration of the study. During storage, as expected, the pH slowly decreased as the microbial population increased until day 8, and thereafter remained constant until the end of the study. Under aerobic conditions, the pH of the 1% NaCl bologna product decreased from 6.1 to 5.9, in the 2% NaCl bologna product the pH changed from 6.2 to 5.7 and in the 3% NaCl bologna product, the pH decreased from 6.2 to 5.9. Under vacuum-packaged conditions, the pH in the 1% NaCl bologna product remained constant at 5.9; whereas, in 2% and 3% NaCl bologna product, the pH decreased from 6.2 to 5.9.

3.4.1.3. Ion Torrent analysis

As a preliminary study, in order to determine the potential for using Ion Torrent sequence analysis for examining of cooked bologna microbial communities, six random samples were taken at day 2 (aerobic packaged, 1% NaCl; vacuum packaged 1% NaCl; aerobic packaged 3% NaCl) and at day 8 (aerobic packaged, 2% NaCl; vacuum packaged 1% NaCl; vacuum packaged 3% NaCl) and the data obtained was pooled for the analysis. In this part of the study, analysis revealed the extent of the diversity of the cooked bologna microflora, as shown in Figure 3.4.

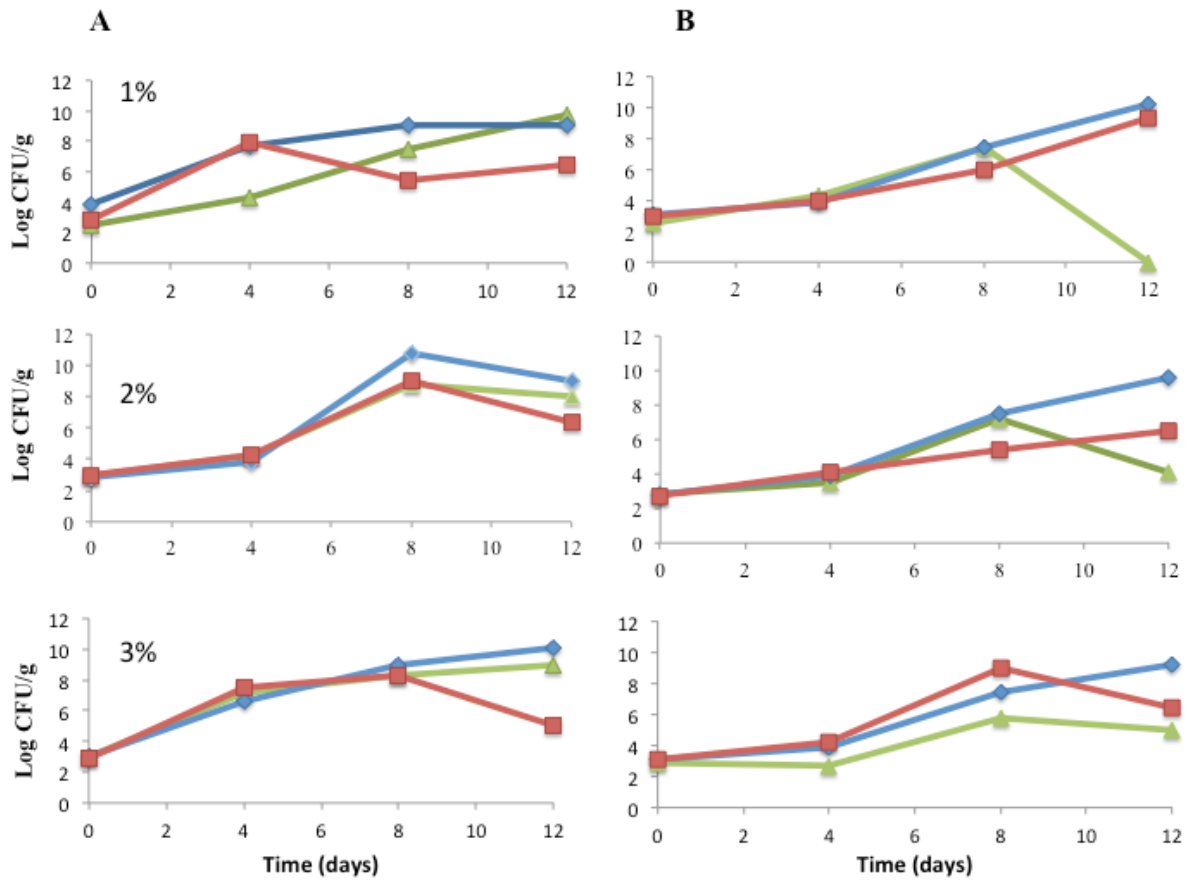


Figure 3.3 Growth of LAB (♦), *P. fluorescens* (▲) and *B. thermosphacta* (■) on sliced cooked bologna formulated with 1, 2 and 3% NaCl and stored under (A) aerobic and (B) vacuum-packaged conditions over 12 days at 8°C.

It was expected that for samples stored over longer periods of time and at lower salt concentrations, the diversity of the product microflora would be greater. Accordingly, the presence of different genera such as *Acidovorax*, *Serratia*, *Staphylococcus* and *Salmonella*, were observed, in addition to the genera which were used as inocula.

Overall, this preliminary study revealed that microbial growth occurred so rapidly that the impact of storage and salt concentration on bacteria growth could not be ascertained. Different studies have focused on the study of multiple variables that affect microbial growth such storage conditions, pH, sodium and nitrate concentrations and temperature (Ratkowsky *et al*., 1982; Zurera-Cosano *et al*., 2006) and most of these results have demonstrated that temperature is the most important factor affecting the rate of microbial growth. High temperatures, and the resultant rapid growth, can interfere with the hurdle effect of the different variables controlled, in this case

salt concentration. In order to reduce the growth rate so that the salt concentration effect could more readily be determined, a storage temperature reduction to 4°C was selected.

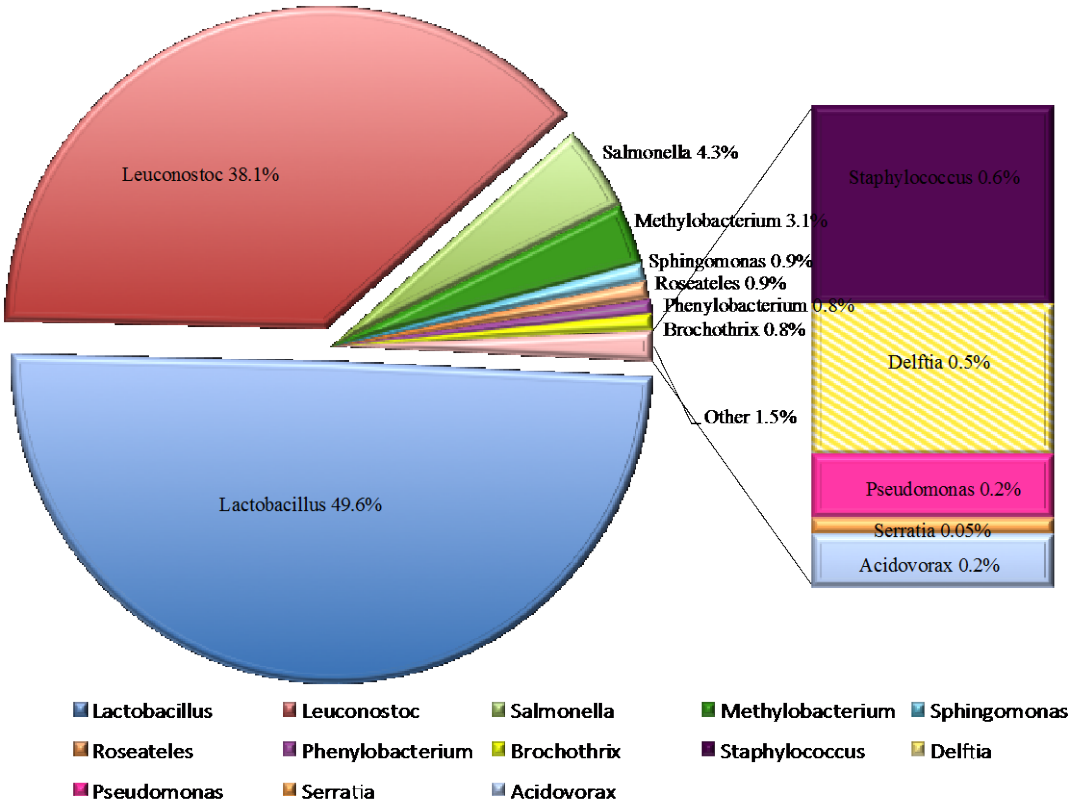


Figure 3.4 Ion Torrent sequence data showing frequency (%) of genera detected.

3.4.1.4. Turbidimetric assay

Preliminary growth determinations were conducted in liquid broth culture to evaluate the general effect of salt on the growth rates of bacteria used as inocula in this study. These experiments were conducted using turbidimetric methods as an indirect method for obtaining information that is proportional to the increase in cell biomass that accompanies cell growth. Beer's Law states that absorbance is proportional to bacteria concentration. For instance, as the bacterial population increases, the transmitted light decreases and the absorbance reading increases on the spectrophotometer in direct proportion with cell growth (Dalgaard *et al*., 1994).

In this study, to determine bacterial growth rates, optical density (OD) values obtained over time for each microorganism were graphed (consisting of x-y pairs of elapsed time and natural log conversion of the OD data). The growth rate for each data set was determined as the

slope of the line generated by the data points used and a faster growth was reflected by the higher slope value. The specific growth rates determined for the different organisms grown under different salt concentrations are shown in Table 3.3. It was observed that growth rate diverged between organisms under the same environmental conditions. Accordingly, it was seen that *L. monocytogenes* had the lowest growth rate relative to the other species; whereas, *L. curvatus* was the microorganism with highest growth rates. *Lactobacillus curvatus* was also the only microorganism affected by the sodium concentration ($p<0.05$).

Table 3.3 Maximum specific growth rates (μ_{max}; hr^{-1}) estimated for different bacteria as a function of the final salt concentration of 1, 2 and 3% (NaCl) at 15°C

Microorganism	Mean ± SD		
	1% NaCl	2% NaCl	3% NaCl
Brochothrix thermosphacta	0.17 ± 0.01	0.16± 0.05	0.16 ± 0.03
Pseudomonas fluorescens	0.15 ± 0.01	0.15 ± 0.06	0.14 ± 0.01
Leuconostoc mesenteroides	0.14 ± 0.02	0.14 ± 0.02	0.14 ± 0.01
Lactobacillus curvatus	0.29 ± 0.02	0.31 ± 0.02	0.28 ± 0.01
Listeria monocytogenes	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.02

* Values are means of three replicates

The doubling time of a culture is known as the definable period during which the cell population double in size and it can be calculated based on the growth rate. For instance, from the specific growth rates (μ_{max}), it was determined that *L. monocytogenes* had the longest doubling time, as shown in Table 3.4. On the other hand, it was possible to observe that salt concentration had the greatest effect on *L. curvatus* growth, resulting in the shortest doubling times when compare to the other microorganisms studied.

Table 3.4 Doubling times for different bacteria in broth formulated with a final concentration of 1, 2 and 3% NaCl at 15°C.

Microorganism	Doubling time, t_D ($t_D = \ln 2/\mu$) (h)		
	1% NaCl	2% NaCl	3% NaCl
Brochothrix thermosphacta	4.1 ± 0.4	4.4 ± 0.2	4.3± 0.7
Pseudomonas fluorescens	4.5 ± 0.4	4.9 ± 1.7	4.9 ± 0.4
Leuconostoc mesenteroides	4.9 ± 0.5	4.9 ± 0.8	4.8 ± 0.2
Lactobacillus curvatus	2.3 ± 0.2[a]	2.3 ± 0.2[a]	2.5 ± 0.1[b]
Listeria monocytogenes	6.8 ± 0.8	7.4 ± 0.4	6.4 ± 0.9

*Means with the same letter are not significantly different ($p < 0.05$)
** Values are means of three replicates ± standard deviation

The effect of NaCl on the growth and metabolism of lactobacilli has previously been reported (Rozes and Perez, 1996; Verluyten *et al*., 2004). In agreement with the results presented here, Verluyten *et al.* (2004) reported that the growth of *L. curvatus* was not affected at low salt concentrations (<2% NaCl). In fact, in a study conducted by Korkeala (1992), it was found that a concentration of 1 and 2% NaCl increased bacteria growth compare to a 0% salt concentration, an observation that can be explained by the fact that *L. curvatus* is quite salt tolerant and commonly found in processed meat. On the other hand, both studies reported that there was a linearly-inhibitory growth effect as sodium concentration was increased. In the experiment conducted by Korkeala (1992), a sodium concentration above 3% was demonstrated to negatively affect bacterial growth, in agreement with the growth rates results shown here.

3.4.2. Enumeration of *Brochothrix thermosphacta* and LAB

Based on the results of the preliminary study, the bologna product incubation protocol was modified to include a storage temperature of 4°C to reduce the rate of microbial growth.

A simple reduction in salt content, without the addition of any other ingredient that compensates for the sodium reduction, is the most direct and realistic way to manufacture meat products low in salt. To investigate the effects of salt reduction on microbial growth behavior, three sodium concentrations were chosen for use in this study: a high sodium concentration (3% NaCl), a reference sodium level equivalent to the salt concentration commonly used in current retail meat products (2% NaCl), and a reduced sodium level (1% NaCl).

The conditions imposed on the microbial spoilage community by the physical and chemical factors predominant in foods influence the selection of microorganisms and define their interactions. Microbial dynamics of LAB and *B. thermosphacta* in the bologna model product with the three different salt formulations under aerobic and vacuum-packaged storage conditions are shown in Table 3.5. Results demonstrated that both LAB and *B. thermosphacta* grew equally well under aerobic conditions; LAB counts increased from 2.9 ± 0.2 to 8.7 ± 0.6 log CFU g^{-1} over the 30 day storage time course; whereas, *B. thermosphacta* increased from 2.8 ± 0.2 to 9.0 ± 0. 5 log CFU g^{-1} (Fig 3.5).

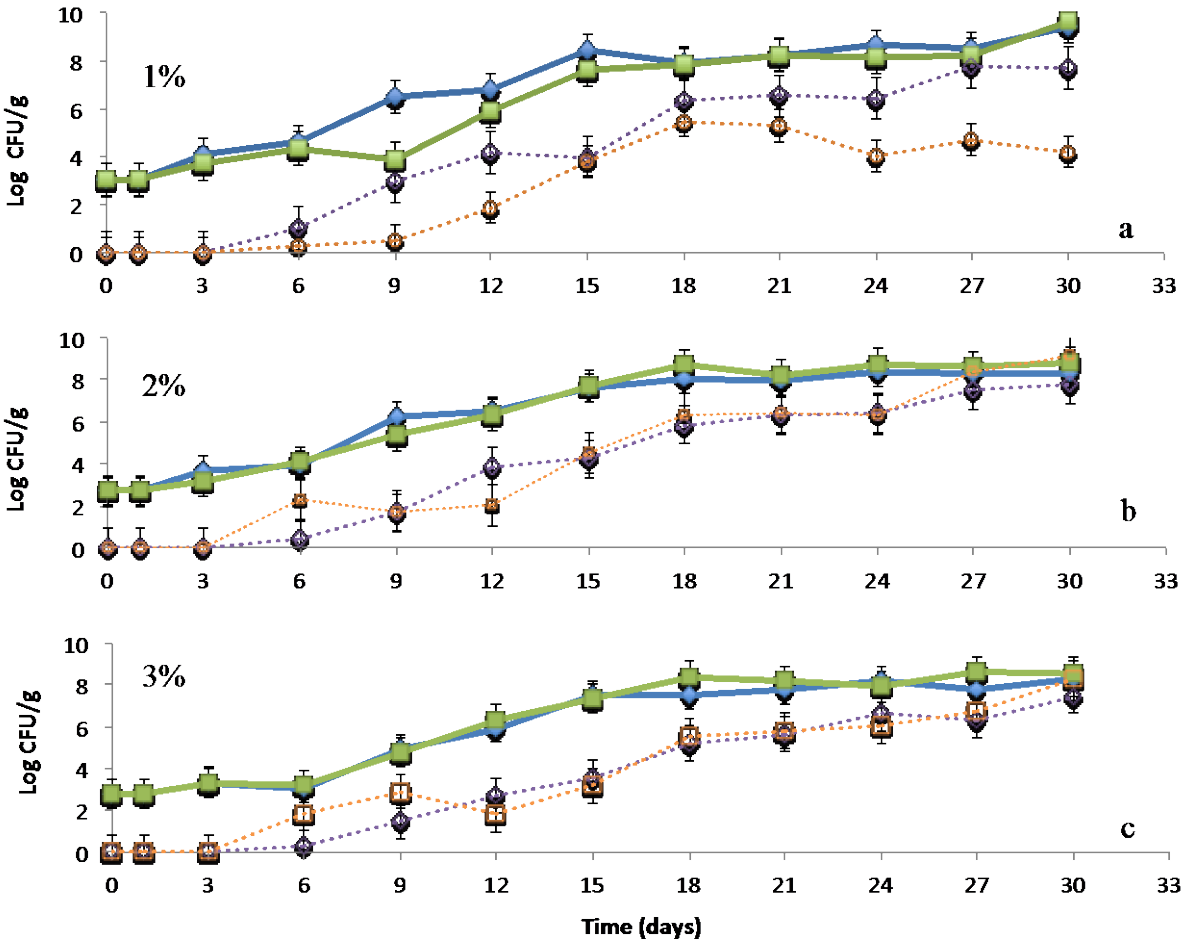


Figure 3.5 Growth response (Log CFU g^{-1}) of LAB and *B. thermosphacta* ((LAB (♦), LAB on uninoculated bologna (◊), *B. thermosphacta* (■), *B. thermosphacta* on uninoculated bologna (□)) averaged from two independent experiments conducted on sliced cooked bologna with 1% (A), 2% (B), and 3% (C) NaCl under aerobic conditions over 30 d at 4°C.

Table 3.5 Viable counts (log CFU g^{-1}) of spoilage bacteria on sliced cooked bologna stored under aerobic or vacuum-packaged conditions at 4°C. Standard deviations were calculated from two independent experiments

Salt (%)	Day	Log CFU g^{-1} ± SD			
		B. thermosphacta		Lactic acid bacteria	
		Aerobic	Vacuum	Aerobic	Vacuum
	0	2.9 ± 0.5	3.5 ± 0.3	2.9 ± 0.6	3.4 ± 0.03
1%	**3**	3.7 ± 0.6	3.2 ± 0.4	4.1 ± 0.8	4.7 ± 1.5
	6	4.3 ± 1.5	3.4 ± 0.3	4.6 ± 0.1	5.0 ± 1.2
	9	3.9 ± 1.1	4.7 ± 0.2	6.4 ± 1.3	6.0 ± 1.3
	12	5.9 ± 1.1	5.6 ± 0.7	6.8 ± 0.1	6.1 ± 1.0
	15	7.6 ± 0.3	6.9 ± 1.2	8.3 ± 0.0	8.1 ± 0.3
	18	7.8 ± 1.3	6.9 ± 0.8	7.9 ± 0.5	7.2 ± 0.2
	21	8 ± 1.2	7.9 ± 0.04	8.2 ± 1.1	8.0 ± 0.6
	24	8.0 ± 0.5	7.0 ± 0.6	8.5 ± 1.1	7.9 ± 0.4
	27	8.0 ± 0.2	7.7 ± 1.7	8.5 ± 0.9	8.0 ± 0.1
	30	8.5 ± 1.0	7.2 ± 2.0	8.8 ± 0.2	8.5 ± 0.1
2%	**0**	2.7 ± 0.4	2.9 ± 0.1	2.7 ± 0.5	2.0 ± 0.2
	3	3.2 ± 1.2	2.6 ± 0.4	3.7 ± 0.4	3.8 ± 2.3
	6	3.8 ± 1.5	3.4 ± 0.2	3.8 ± 0.2	4.4 ± 1.0
	9	5.2 ± 0.4	3.9 ± 0.7	6.1 ± 1.4	5.5 ± 0.5
	12	6.3 ± 0.2	4.6 ± 2.5	6.4 ± 0.2	5.8 ± 0.9
	15	7.7 ± 0.1	6.0 ± 1.1	7.6 ± 0.4	8.0 ± 0.5
	18	6.2 ± 0.2	6.5 ± 1.7	8.0 ± 0.4	7.8 ± 0.1
	21	8.1 ± 1.1	7.4 ± 1.8	7.8 ± 0.2	7.4 ± 0.2
	24	8.5 ± 0.7	6.7 ± 2.1	8.3 ± 0.1	7.5 ± 0.5
	27	8.5 ± 0.2	6.9 ± 1.8	8.3 ± 0.1	7.6 ± 0.5
	30	8.7 ± 0.1	7.1 ± 2.5	8.2 ± 0.4	8.1 ± 0.5
3%	**0**	2.8 ± 0.2	2.7 ± 0.4	3.0 ± 0.5	3.0 ± 0.4
	3	3.3 ± 0.1	2.4 ± 0.1	3.2 ± 0.6	3.2 ± 0.4
	6	3.4 ± 0.1	2.9 ± 0.1	2.9 ± 0.3	3.3 ± 0.4
	9	4.7 ± 1.7	3.2 ± 0.8	4.9 ± 0.1	4.1 ± 1.4
	12	6.3 ± 0.1	3.4 ± 1.7	5.9 ± 0.2	3.4 ± 0.6
	15	6.9 ± 1.7	5.2 ± 1.7	7.3 ± 1.1	4.7 ± 0.4
	18	8.4 ± 0.6	6.2 ± 1.7	7.5 ± 0.9	5.7 ± 2.1
	21	8.1 ± 0.2	6.4 ± 1.9	7.7 ± 0.6	6.0 ± 0.3
	24	8.1 ± 0.3	6.9 ± 1.8	8.1 ± 0.6	5.8 ± 0.2
	27	8.4 ± 0.2	7.4 ± 1.7	7.7 ± 0.9	5.8 ± 0.1
	30	8.2 ± 0.1	7.5 ± 1.7	7.9 ± 0.5	6.0 ± 0.4

*Values are means ± standard deviation

Regarding uninoculated treatments, it was possible to determine the presence of LAB and *B. thermosphacta*, confirming these microorganisms are part of the spoilage process of cooked meats. When comparing the growth of spoilage bacteria on uninoculated to growth on inoculated bologna, it was possible to observe that even though bacteria numbers were below the detection limit of the media implemented, LAB and *B. thermosphacta* reached high numbers as was observed on inoculated bologna.

When comparing the growth of different spoilage bacteria on inoculated bologna under aerobic conditions, no antagonistic effect of LAB on *B. thermosphacta* was evident, and after 15 days of storage, both microorganisms increased in number to over 7 logs CFU g^{-1} and kept growing until the end of the study. The lack of any antagonistic interaction between these two spoilage bacteria is supported by the results of Cocolin *et al.* (2004), who found that the numbers of *Leuc. mesenteroides* and *B. thermosphacta* remained high right until the end of their study, and concluded these two organisms were the most active spoilage organisms in their system. On the other hand, when analyzing the effect of sodium on microbial growth, it was possible to determine that contrary to *B. thermosphacta*, growth of LAB was significantly reduced at the highest concentration of salt ($p<0.05$), as shown in Table 3.5. The results from the current study agree with those obtained by Korkeala *et al*. (1992) who reported that a salt concentration of 3% affected LAB growth and that under this condition, *Leuconostoc* sp. tended to be more sensitive than *Lactobacillus curvatus.*

As shown in Table 3.6, statistical analysis showed that LAB were significantly affected by the highest concentration of salt and under this condition, growth was also affected by the packaging conditions (aerobic versus vacuum packaging). However, when sodium concentrations of 1% and 2% salt were compared, no salt effect or storage condition effect were found. Alternatively, statistical analysis of *B. thermosphacta* cell numbers revealed that the only factor affecting microbial growth was storage condition (aerobic versus vacuum packaging).

The effect of different packaging conditions on microbial growth and how these conditions select for different spoilage bacteria has also been the focus of other studies (Cayré *et al.*, 2005; Vermeiren *et al.,* 2005). Accordingly, the shelf-life of meat products and cooked ham has been demonstrated to be considerably improved by vacuum packaging, providing up to 3 to 4 weeks storage life at storage temperatures close to 0°C (Labadie, 1999; Vercammen *et al.*, 2011).

Table 3.6 *P*-value for estimated parameters for LAB and *B. thermosphacta* growth in sliced cooked bologna

Effect	Probability	
	LAB	***B. thermosphacta***
Salt	0.0009	0.5851
Packaging condition	0.0216	0.0263
Day	<.0001	<.0001
Salt*Day	0.3033	0.9498
Condition*Day	0.0661	0.4121

Product deterioration is commonly associated with the presence of lactic acid bacteria; studies have reported that under low oxygen conditions and at low temperature, LAB are the dominant spoilage group (Mayr *et al.*, 2003; Nowak and Krysiak, 2005; Signorini *et al.*, 2006) and growth of *B. thermosphacta* is generally reduced. Nevertheless, the present study revealed that *B. thermosphacta* numbers reached over 7 log CFU g^{-1} on bologna formulated with 3% salt (compared with over 9 log CFU g^{-1} on 1 - 2% NaCl bologna) (Fig 3.6).

In the present study, *B. thermosphacta* was found to be a numerically significant component of the spoilage microflora and it was not inhibited nor dominated by LAB. This result was expected, due to the fact the growth and metabolism of this microorganism depend on factors such as pH of about 5.7 (Grau, 1980), nutrient availability and absence of oxygen (Gribble *et al.*, 2014), conditions that were not achieved in this study.

On the other hand, independent of sodium concentration, LAB significantly increased with time ($p<0.05$) as expected, from 2.71 ± 0.31 log CFU g^{-1} to 9.0 ± 0.68 log CFU g^{-1} on 1% - 2% NaCl, and to 6.38 ± 0.8 on 3% NaCl. Finally, regarding the effect of packaging conditions, it was determined that storage conditions influence LAB growth ($p<0.05$).

3.4.3. Enumeration of *Pseudomonas fluorescens*

As reported in other studies, the aerobic spoilage flora of fresh and cooked meat products stored at chill temperatures (4°C) is usually dominated by *Pseudomonas* spp. (Labadie, 1999; Russo, 2006; Aaslyng, 2014). There have also been various studies reporting the effects of temperature, oxygen availability and water activity on *Pseudomonas* spp. prevalence (Labadie, 1999); however, there is scarce information of this organism, overall, with respect to salt tolerance, especially on cooked meat products (Durack *et al.*, 2013).

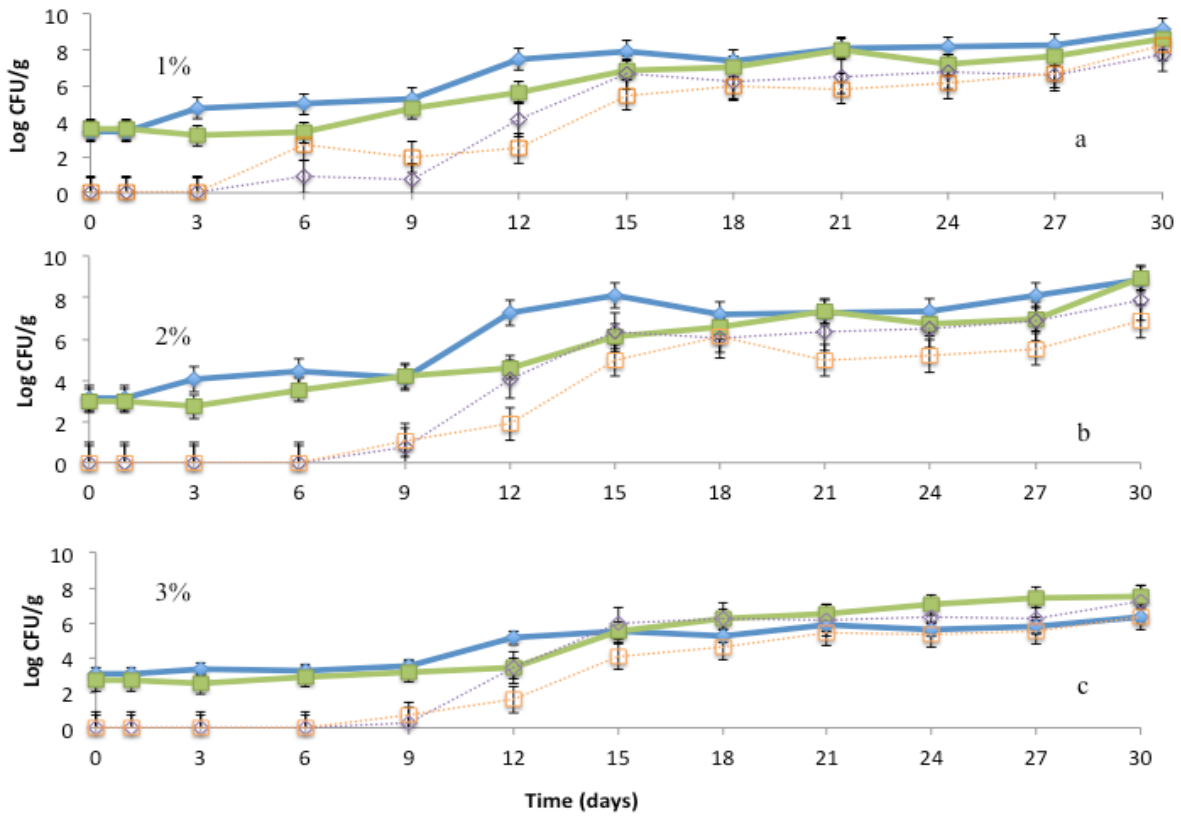


Figure 3.6 Growth response (Log CFU g^{-1}) LAB and *B. thermosphacta* ((LAB (♦), LAB on uninoculated bologna (◊) *B. thermosphacta* (■), *B. thermosphacta* on uninoculated bologna (□)) averaged from two independent experiments conducted on sliced cooked bologna with 1% (A), 2% (B), and 3% (C) NaCl under vacuum conditions 30 d at 4°C.

In Trial 1 of this project, significant bacterial growth was observed under aerobic conditions, with cell numbers increasing from a starting concentration of 2.79 ± 0.12 log CFU g^{-1} to a final concentration of 9.04 ± 0.12 log CFU g^{-1} by day 30 (Fig 3.7). Regarding the effect of salt, there was no significant difference ($p = 0.05$) on *P. fluorescens* growth between salt treatments, a result that supports both the findings from the turbidimetric growth assay as well as findings recently reported by Aaslyng (2014); whereas, a significant effect of storage condition and day on bacterial growth was assessed ($p<0.05$) along with a significant interaction observed between salt and day ($p<0.05$). Furthermore, there was a significant interaction between condition and day ($p<0.05$) on bacterial growth results due to the fact vacuum packaging retarded *P. fluorescens* growth (Table 3.7).

Regarding uninoculated bologna, it was possible to detect and confirm the presence of *P. fluorescens* on cooked bologna. Under aerobic conditions in Trial 1, there was an increase in *P. fluorescens* numbers, as expected, independent of salt concentration use in bologna formulation; spoilage bacteria reached high numbers of about 7.1 ± 0.8 log CFU g^{-1}, 6.9 ± 0.43 log CFU g^{-1} and 7.6 ±0.33 log CFU g^{-1} on 1%, 2% and 3% NaCl, respectively.

Based on the high numbers obtained in uninoculated bologna, it is possible to say that the high numbers obtained on inoculated bologna throughout the study might be due to the high initial numbers of *P. fluorescens* as part of the native microflora.

Table 3.7 *P*-value for estimated parameters for *Pseudomonas fluorescens* growth in sliced cooked bologna

Effect	Trial 1	Trial 2
Salt	0.05	0.0002
Packaging condition	0.0001	<.0001
Day	<.0001	<.0001
Salt*Day	0.003	<.0001
Condition*Day	<.0001	<.0001

Interestingly, *P. fluorescens* growth results from Trial 2 were significantly different from Trial 1. During trial 2, there was an increase in *P. fluorescens* cell numbers from 2.28 ± 0.2 log CFU g^{-1} to 4.9 ± 0.8 log CFU g^{-1} by day 15, and thereafter followed by a decline in cell numbers until the study's end.

Contrary to Trial 1, initial numbers of indigenous *P. fluorescens* on uninoculated bologna were lower than in Trial 1. In Trial 2, maximum *P. fluorescens* numbers of 3.3 ± 1.3 log CFU g^{-1} by day 18 on 1% NaCl, 2.3 ± 0.58 log CFU g^{-1} by day 21 on 2% NaCl and 1.5 ± 1.3 log CFU g^{-1} by day 21 on 3% NaCl, were observed.

Statistical analysis of data obtained from Trial 2 demonstrated microbial growth was strongly affected by storage condition, day, the interaction between salt and day, condition and day and finally salt, condition and day ($P<0.001$).

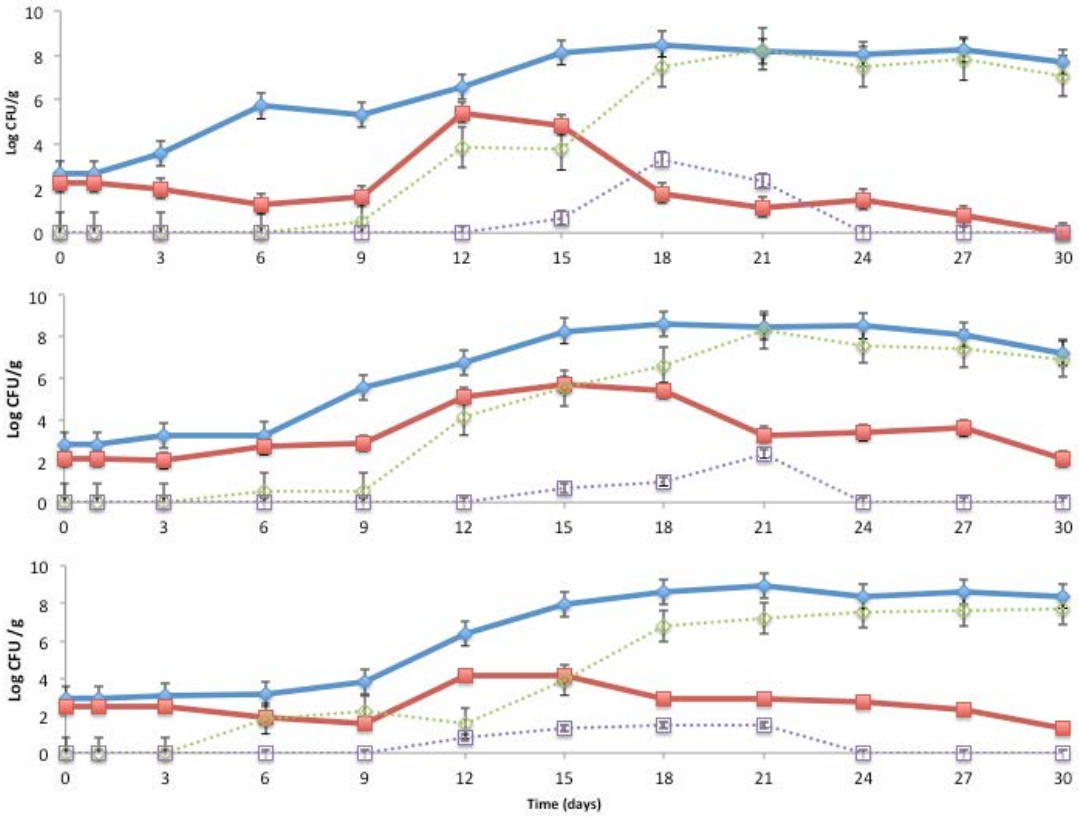


Figure 3.7 Growth response (Log CFU g^{-1}) of *P. fluorescens* (*P. fluorescens* on uninoculated bologna Trial 1 (◊) *P. fluorescens* Trial 1 (♦), *P. fluorescens* on uninoculated bologna Trial 2 (□), *P. fluorescens* Trial 2 (■) conducted on sliced cooked bologna with 1% (A), 2% (B), and 3% (C) NaCl under aerobic conditions 30 d at 4°C.

Regarding salt concentration, this analysis demonstrated there was also a significant effect of salt ($p<0.05$) on microbial growth, more specifically, a sodium concentration of 2% had a significant effect on microbial cell numbers when compared to 1% and 3% NaCl ($p<0.05$) sodium concentration. This may be due to the fact that bacterial numbers on 2% NaCl were higher than on bologna formulated with 1 and 3% NaCl, and also reached the minimum detection limit more slowly compared to the other two salt treatments. This result was not expected as *P. fluorescens*, which is an aerobic microorganism, typically reaches higher population numbers under aerobic conditions, as observed in other studies (Tsigarida *et al*., 2000; Liu *et al*., 2006), and as observed during Trial 1.

This unexpected result under aerobic conditions may be explained by the reducing conditions of the environment. *Pseudomonas fluorescens* is an aerobe and as such, its growth would be expected to occur on the surface of the sliced product where the environment is more oxidizing. As it is shown in section (3.3.10), the E_h on the surface decreased more compared to measurements obtained from between the slices with E_h values approximating -50 mV by day 7.

Another factor that may have influenced these unexpected results is the concentration of indigenous microorganism. In the days prior to preparing the bologna used in Trial 2, the meat processing plant went through a series of repairs, along with an intensive cleaning and sanitation process, which likely reduced the initial bacterial levels in the bologna.

As mentioned previously, vacuum packaging was expected to retard *P. fluorescens* growth in comparison to aerobic packaging. However, major differences in numbers were observed when comparing Trial 1 to Trial 2. For example, it was possible to observe bacteria in Trial 1 reaching a maximum cell density of 8 log CFU g^{-1} by the end of the study; whereas, in Trial 2 after 24 days, viable *P. fluorescens* cells could no longer be detected (Fig 3.8). The gaseous phase composition of vacuum-packaged meats has been reported to change over storage time. Accordingly, it would be expected that the concentration of oxygen would decrease while carbon dioxide would increase, favoring and selecting for bacterial flora able to tolerate CO_2 like LAB (Ercolini *et al.*, 2006).

The growth of *P. fluorescens* during the first 9 days of Trial 2 is in agreement with the results reported by Babji *et al.* (2000), who observed an initial increase in total aerobic bacteria as well as Pseudomonad numbers over the first week of storage due to the fact that conditions were not completely anoxic during this period of time. As the gas composition changes, the oxidizing-reducing value of the environment also changes; an E_h of +40 mV has been the minimum value reported to support *P. fluorescens* growth under low oxygen conditions at pH 7 (Tabatabai and Walker, 1970).

The unusual growth pattern of *P. fluorescens* cells during Trial 1 suggests that there were apparently significant technical issues that occurred, most likely during the process of packaging. This may have included the inadequate sealing of the packages, which resulted in oxygen penetration during the storage period, thereby enabling the growth of this strictly aerobic bacterium. It has been reported environmental redox potential is an important determinant of microbial growth, acting as a selective agent and influencing metabolic microbial product

(ICMSF, 1980). Thus, it is also possible to assume that the bologna used in Trial 2 was somehow was more reducing and anaerobically-poised, thus resulting in the observed rapid decline of *P. fluorescens* numbers under both aerobic and anaerobic conditions.

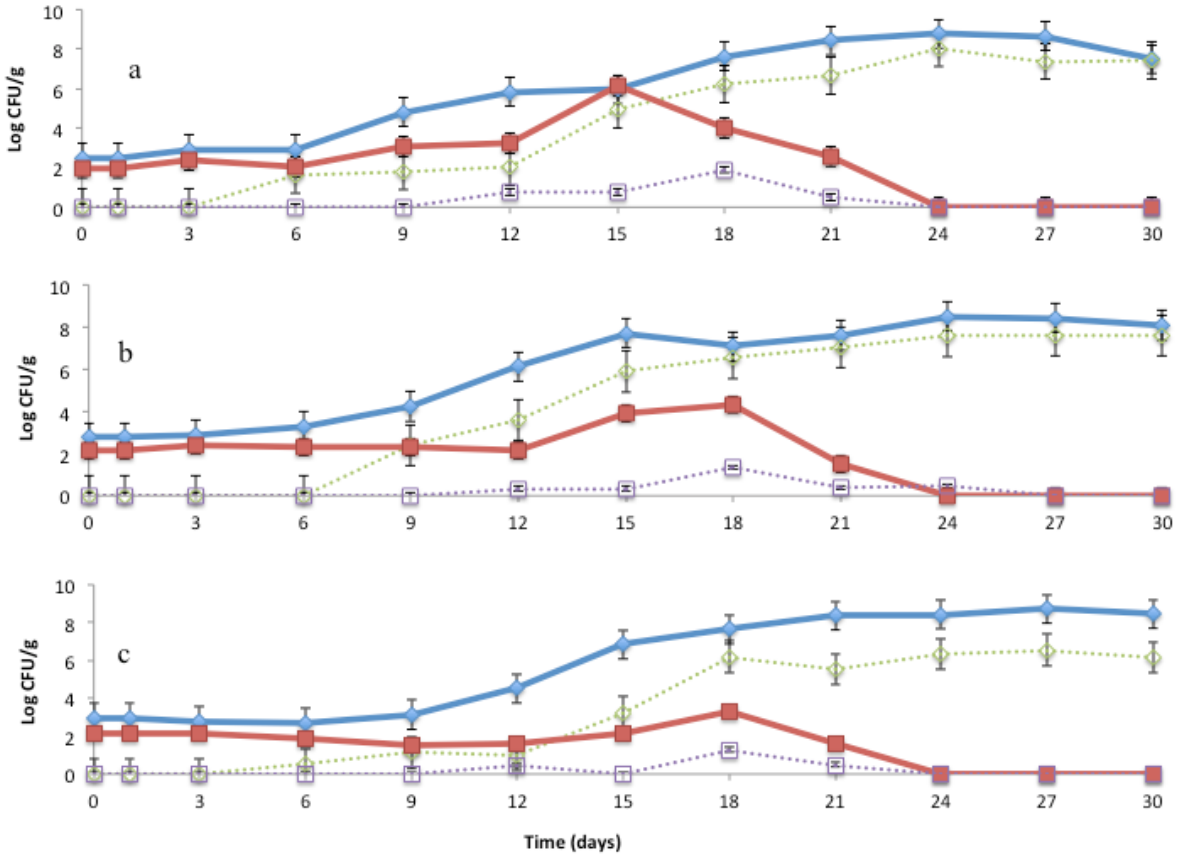


Figure 3.8 Growth response (Log CFU g^{-1}) of *P. fluorescens* (*P. fluorescens* on uninoculated bologna Trial 1 (◊) *P. fluorescens* Trial 1 (♦), *P. fluorescens* on uninoculated bologna Trial 2 (□), *P. fluorescens* Trial 2 (■) on sliced cooked bologna with 1% (A), 2% (B), and 3% (C) NaCl under vacuum conditions at 4°C.

3.4.4. Culture-independent community identification

Next-generation sequencing techniques have been extensively used to study environmental ecosystems (Shokralla *et al.*, 2012). However, its potential to study food microbial communities hasn't been widely explored. The Ion Torrent platform is, to date, one of the lowest cost next

generation sequencing platforms that allow processing of millions of reads in a single run (Whiteley *et al*., 2012).

Several researchers have previously described spoilage organisms in meat products by using molecular techniques such PCR-DGGE (Hu *et al.,* 2009; Tu *et al.,* 2010). The domain *Bacteria* has been grouped into 23 phyla; an only microorganism belonging to the phyla *Proteobacteria, Firmicutes, and Actinobacteria* have been reported extensively in meat products; whereas, microorganisms belonging to the group *Bacteroidetes* have been less reported.

The Gram-negative bacteria of known importance in foods belong to the class *Proteobacteria*. The class is divided into five subclasses *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria,* and *Epsilonproteobacteria*. They include a variety of pathogens, such as *Escherichia, Salmonella, Vibrio* and *Helicobacter* (Jay *et al.,* 2004).

Firmicutes is one of the main phyla within the *Bacteria* and currently it encompass three classes: *Clostridia, Mollicutes* and *Bacilli*, being the last one represented by lactic acid bacteria (LAB), including *Lactococci, Lactobacilli, Leuconostoc, Weissella, Carnobacteria,* and *Enterococci*. (Haakensen *et al*., 2008; Remenant *et al*., 2014).

The phylum *Actinobacteria* is one of the largest phyla within the domain *Bacteria*, with 39 families and 130 genera (Goodfellow, 2012; Ventura *et al.,* 2007). Microorganisms within this group are characterized for comprising gram-positive organisms with a high G+C composition in their DNA (> 50% G+C) and exhibit a tendency towards mycelial growth (Ventura *et al.*, 2007; Goodfellow, 2012). This phylum is comprised of pathogenic microorganisms, such as *Corynebacterium, Mycobacterium* and *Nocardia*, soil inhabitants, like *Streptomyces*, or gastrointestinal commensals such *Bifidobacterium* (Stackebrandt and Schumann, 2006; Ventura *et al.*, 2007).

The bacteria belonging to *Bacteroidetes* (previously known as the *Cytophaga-Flavobacteria-Bacteroides* (CFB) group) are the major members of the microbiota of animals, especially in the gastrointestinal tract. These microorganisms can act as pathogens and are frequently found in soils, oceans and freshwater. The phylum *Bacteroidetes* comprises four classes: *Bacteroidia, Flavobacteria, Cytophagia* and *Sphingobacteria*. These bacteria are all Gram-negative; they can be strictly anaerobic like *Bacteroides* or strictly aerobic like *Flavobacteria*. They are non-motile, flagellated, or move by gliding (Ludwing *et al.,* 2011;

Thomas *et al.*, 2011)

In this study, by using Ion Torrent sequencing, it was possible to capture a snapshot of microbial diversity and abundance of the microbiome. A total of 37,295 and 40,445 quality-trimmed 16S rRNA gene amplicon sequences were obtained from 1% and 3% NaCl cooked bologna formulae after 15 days of growth, respectively.

In general, day 15 16S rRNA gene libraries showed that bacteria community was comprised primarily of four phyla: *Firmicutes, Proteobacteria, Actinobacteria* and *Bacteroidetes*. Libraries that represented less that 1% of abundance were classified as "others". Inoculate bologna samples formulated with 1% NaCl were dominated by the phylum *Firmicutes* regardless of the storage condition; whereas on uninoculated bologna formulated with 1% NaCl, phylum *Firmicutes* dominated with an average of 74.1%, the phylum *Actinobacteria* was detected at a frequency of 9.25% and the phylum *Proteobacteria* was detected at a frequency of 20.1% only on bologna stored under aerobic conditions. Finally, *Bacteroidetes* was detected in a low frequency of 0.1 and 2.1 % on bologna under vacuum and aerobic conditions, respectively (Figure 3.9).

On the other hand, microbial community of inoculated bologna formulated with 3% NaCl, was represented by phylum *Firmicutes* comprising 100% and 16.6% of the sequence reads in bologna stored under aerobic and vacuum packaged conditions, respectively. *Proteobacteria* was the second major phylum detected; in bologna stored under vacuum conditions it comprised 79.2% of the reads detected. Finally, on uninoculated bologna, phylum *Firmicutes* represented the 97 and 88.5 % of the total sequences, phylum *Bacteroidetes* was detected in a low frequency of 0.1 and 1 % and *Proteobacteria* was detected at a frequency of 2 and 8% on bologna stored under aerobic and vacuum packaging conditions, respectively.

The microbial diversity observed at the order level is reported in Figure 3.10. On samples formulated with 1% NaCl, 7 orders were identified. Although there were unique bacteria in all bologna samples, the most abundant bacteria belonged to the orders *Bacillales* and *Lactobacillales*. As expected, under aerobic conditions, the order *Bacillales* was most abundant, comprising 95% of the detected sequences; whereas, under vacuum conditions, its incidence was reduced to 65% as the presence of *Lactobacillales* increased.

When comparing uninoculated treatments (e.g., indigenous bologna microflora), it was possible to observed considerably more bacterial diversity, beyond that of LAB and *Brochothrix*, on cooked bologna stored under aerobic conditions. Microorganisms belonging to the family

Carnobacteriaceae, Enterococcaceae, Moraxellaceae, Ruminococcaceae, Pseudomonadaceae, Micrococcaceae and *Bacteroidaceae* were all detected; these sequences were assigned to the genera *Carnobacterium, Enterococcus, Acinetobacter, Psychrobacter, Ethanoligenens, Flavonifactor*, *Pseudomonas, Arthrobacter*, *Bacteroides*, and *Oscillibacter*. A study conducted by Pennacchia *et al.* (2011) revealed the presence of *Psychrobacter* spp. and *Acinetobacter* spp. in fresh meat stored aerobically and have accordingly been reported to be minor agents in the spoilage process. The presence of *Carnobacterium spp*. and *Carnobacterium divergens* have been reported by Ercolini *et al.* (2011) and Vasilopoulos *et al.* (2008) in fresh beef and cooked ham, respectively; whereas, the presence of *Enterococcus faecalis*, *E. hermanniensis* and *E. gilvus* have been identified on cooked ham (Vasilopoulos *et al.*, 2008). Interestingly, *Pseudomonas,* which based on the day 15 viable count data as well as literature, is well-known to be an aerobic spoilage organism, was detected at a low frequency under aerobic conditions in the present study. This result suggests that for some reason, a low recoverability of *Pseudomonas* DNA occurred. It also emphasizes the need for culture-based studies to be conducted in parallel with culture-independent analyses or other positive control due to the fact erroneous conclusion can be reached. In fact, in a study conducted by Khan *et al* (2013) low numbers of *Proteobacteria* by using Ion Torrent were obtained contrasting the high numbers of culturable *Proteobacteria* isolates. Under vacuum packaging conditions the order *Actinomycetales* comprised the 15% of total sequences, representing the genus *Propionibacterium* an anaerobic non-sporulating, Gram-positive bacilli, related to the lactic acid bacteria but not considered to fit the group.

Microbial diversity analyses on bologna formulated with 3% NaCl, revealed the presence of 10 orders in cases where samples obtained from bologna stored under vacuum packaging conditions exhibited the highest diversity of microorganism. *Burkholderiales* appeared to be the predominant order at this sampling point under vacuum packaging conditions, indicating the presence of bacteria derived from environmental origin (Jay *et al.*, 2004). Organisms belonging to the family *Pandorea*, *Oxalobacteriaceae* and *Comamonadaceae* were also detected. The order *Rhizobiales* represented 11% of total sequences on bologna communities colonizing vacuum-packaged 3% NaCl-formulated bologna, and included the genera *Afipia*, *Brucella* and *Bradyrhizobium*.

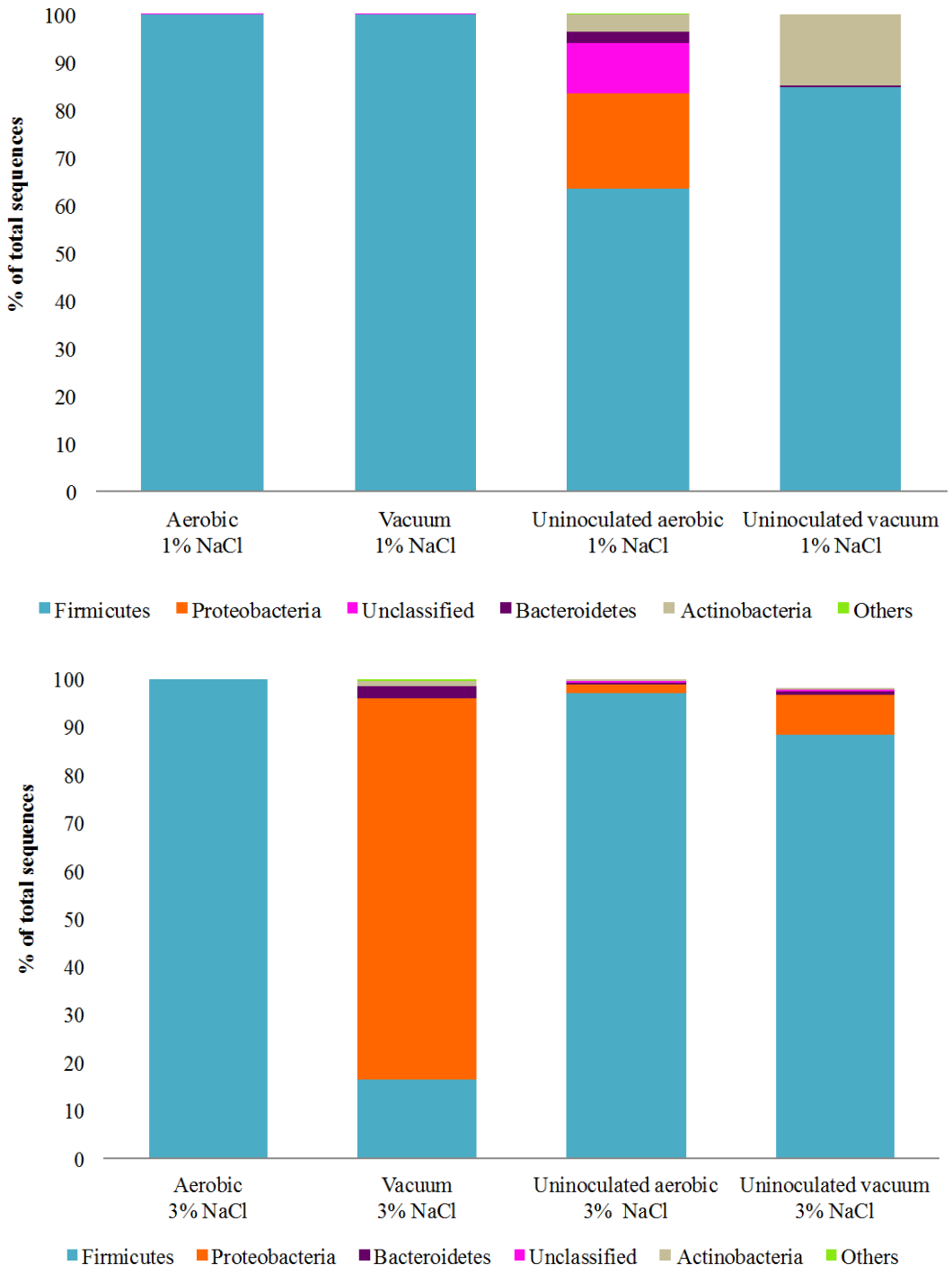


Figure 3.9 Taxonomic classification of bacterial reads at phylum level using RDP classifier with a confidence threshold of 50% retrieved from inoculated and uninoculated sliced cooked bologna formulated with 1% (Top), and 3% (Bottom) NaCl, stored under aerobic and vacuum conditions at 4°C at day 15 in Trial 2.

On the other hand, it was possible to observe that on the 3% NaCl product, most of the bacteria belonged to the *Bacillales* order with the family *Listeriaceae* (which represent the genus *Brochothrix*) surprisingly being most abundant under aerobic conditions, representing on average 69% (± 10.6%) of the population. The second major order in the 3% NaCl system was *Lactobacillales,* especially on uninoculated samples, comprising on average 47% of the total sequences and representing the family *Lactobacillaceae* in 15% (± 10.6%) and the family *Leuconostocaceae* in 5% (± 7%) of the occurrences.

In general, Ion Torrent was used for the first time to assess the structure of microbial communities inhabiting cooked bologna. Over the past few years, microbial community characterization of meat and cooked meat has been accomplished by DGGE analysis. However, DGGE is a time consuming technique and it has been reported that bacteria present in small concentration are often overlooked (Leite *et al.,* 2012).

Even though Ion Torrent is a rapid and accurate technique for analyzing microbial communities, it is important to mention there are some factors that must be considered. Firstly, reads lengths are shorter compared with the sequences obtained with traditional Sanger sequencing methods (600– 800 bp), limiting the classification of the bacteria communities to the genus level (Seong *et al.,* 2010), and secondly, DNA concentration is an important factor that may determine the percentage of sequences obtained from each sample. In this study, DNA may have been lost during the purification process, resulting in the low sequence abundance for certain microorganisms (i.e., Pseudomonads) known to be present.

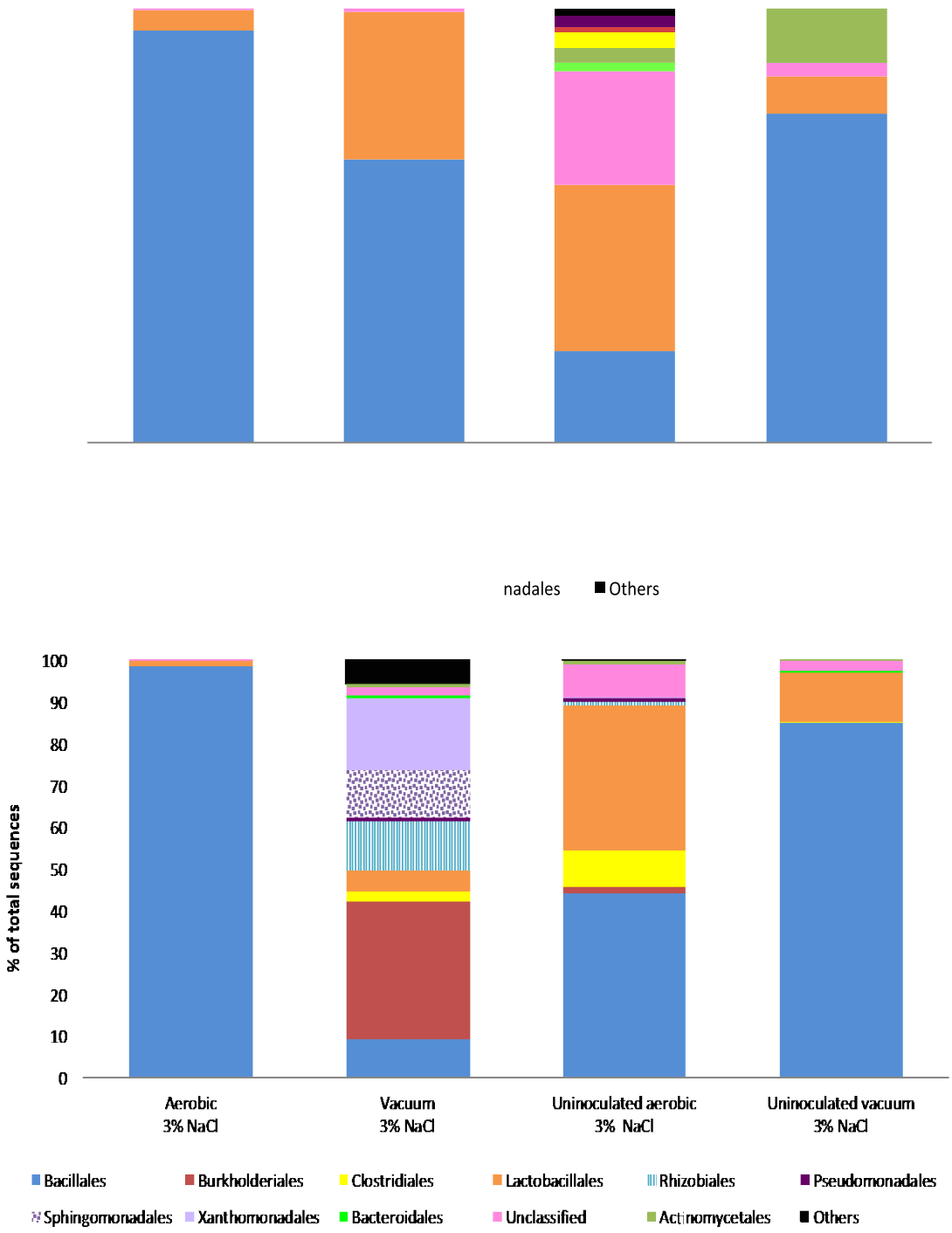


Figure 3.10 Taxonomic classification of bacterial reads at order level using RDP classifier with a confidence threshold of 50% retrieved from inoculated and uninoculated sliced cooked bologna formulated with 1% (Top), and 3% (Bottom) NaCl, stored under aerobic and vacuum conditions at 4°C at day 15 in Trial 2.

3.4.5. Chemical analyses

3.4.5.1. pH and water activity

Salt plays an important role in food preservation by lowering the water activity. In this study, a sodium concentration of 1% NaCl yielded an a_w significantly ($p<0.05$) different from 2% and 3% NaCl (Table 3.8). However, based on the results obtained from viable counts, it is possible to conclude that these variations don't affect microbial growth in this food system.

The pH of the meat products generally decreased with increasing numbers of microorganisms. Under aerobic conditions, the pH of the 1% NaCl cooked bologna decreased from 6.51 to 6.26, remaining more or less unchanged in the 2% NaCl product (decreasing from 6.44 to 6.4), and decreased from 6.44 to 6.3 in the 3% NaCl product. In vacuum-packaged bologna, the pH decreased to 5.4, 5.5 and 5.6 in 1, 2 and 3% NaCl bologna formulations, respectively.

These changes in pH were demonstrated not to affect bacteria growth; *B. thermosphacta* has been reported to grow down to a minimum pH of 4.8 (Leroi *et al.*, 2012) and *P. fluorescens* has been reported to grow at a minimum pH value of 4.5 (Garrity *et al.*, 2005). In fact, growth of *Pseudomonas* causes an increase in pH surface due to proteolytic breakdown of meat proteins. A study conducted by Bala *et al*. (1977) showed that the increase in pH occurred as result from microbial production of ammonia. Hydrogen sulphide, ammonia, indole are produced when *Pseudomonas* utilizes amino acids.

Table 3.8 Effect of sodium concentration on water activity

% NaCl (w/w)	a_w
1	0.9849^{A}
2	0.9803^{B}
3	0.9792^{B}
SEM	0.0016

*Means with the same letter are not significantly different ($p >0.05$).
**Values are means of three replicates

3.4.5.2. Redox potential

Redox potential (E_h) is a physicochemical parameter that defines how oxidized or reduced a particular substance is. From the perspective of food preservation and safety, redox potential measurements offer information that can aid in determining whether a substrate is capable of

supporting growth of various microorganism and also provide an indication of how the background redox potential can be adjusted by the addition of reductants or oxidants in order to make the substrate inhospitable to microorganism (Morris, 2000).

Redox potential measurements from Trial 1 were obtained for cooked bologna held under aerobic and vacuum-packaged conditions at 4°C. As shown in Figure 3.11, the E_h values on cooked bologna stored under aerobic and vacuum-packaged conditions initially were positive (~ 200 mV). However, these values became more negative (~ -150 mV) with time of storage (over the first 6 – 7 days) due to microbial growth under both anaerobic and vacuum conditions, regardless of sodium concentration. Under vacuum-packaged conditions on 1% NaCl, redox was expected to result in more reducing values, reflective of low concentrations of oxygen in the package as a consequence of the reduced antimicrobial/a_w effect of salt on limiting overall microbial growth.

Kukec *et al.* (2002) reported that measurement of redox potential would permit the assessment of the viability of microorganisms, their growth, as well as their physiological activity under defined conditions of environment (temperature, pH, packaging) (Ignatova *et al.*, 2010). When different species of microorganisms are present in a food system, their abilities to change the redox potential and tolerate those values are important considerations in microbial competition and in the establishment of microbial succession (ICMSF, 1980; Morris, 2000).

In this study, the E_h values obtained on the surface of the slices decreased more than between slices in most cases. This is in agreement with Holley (1997), who demonstrated that microbial growth causing spoilage primarily occurred on exposed surfaces of meat products, and that E_h on the surface would be more affected by microbial activity than the E_h in between the slices. In sliced bologna formulated with 3% NaCl and stored aerobically, this trend was not observed for technical reasons; presumably due to the movement of the electrode within the packaged at the moment of packaging or during storage time, or due to the lost of contact between the electrode and the substrate, resulting in unreadable/unusable data.

In general, environmental redox potential has received attention for its potential usage as a determining factor for controlling microbial growth, as E_h can act as a selective agent influencing the metabolic products as well as the type of microorganisms that can proliferate in a given system (ICMSF, 1980). It also offers potential for monitoring microbial growth and metabolic oxygen consumption (i.e., creation of a reducing environment). However, others have

experienced difficulties in obtaining accurate and reproducible readings using electrodes (ICMSF, 1980; Tabatabai and Walker, 1970), reflective of the inconsistent results obtained in the present study. Clearly, more precautions regarding electrode handling and packaging procedures (in order to avoid ingress of air) must be taken into account for this sensitive method to yield consistent data.

Despite potential drawbacks, the measurement of E_h has demonstrated its potential for monitoring microbial growth and spoilage potential in food products. Such real-time information offers a means for monitoring the progression of microbial growth during food spoilage events, and enhances our ability to evaluate appropriate methods for food preservation.

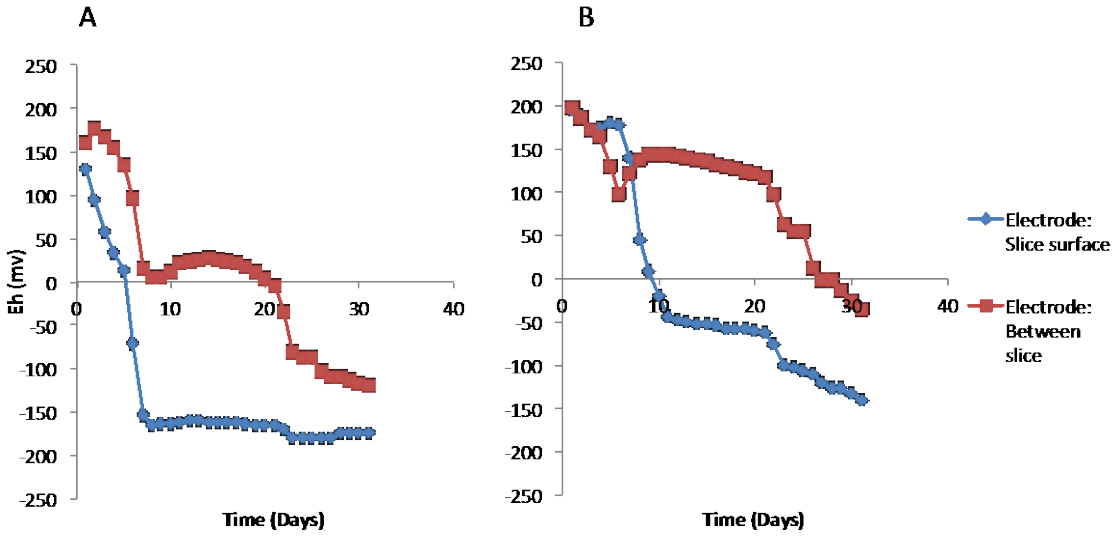
3.5. Conclusions

Based on the results obtained in this study, it is possible to conclude that it is feasible to reduce sodium concentration without the addition of sodium replacements. Several studies (Durack *et al.*, 2013; Aaslung *et al*., 2014) have shown that a sodium reduction has more impact on the sensory properties rather than on microbial growth and safety. In agreement with those previous works, this study found that a sodium reduction do not affect rates of spoilage.

Commercial regular bologna contains ~2% NaCl providing approximately 35% of the allowable daily salt intake (6 g) (Durack *et al*., 2013). In this study, bologna was formulated with 1%, 2% and 3% NaCl which corresponded to a sodium content of 525 mg/100 g, 1071 mg/100 g and 919 mg/100 g bologna, respectively. The 1% bologna formulation would be below the target (655 mg sodium/100 g) serving concentration proposed by Heart and Stroke Foundation (CTAC 2009; 2010).

Understanding the microbial behavior under different storage product conditions is one of the key aspects when determining a product's shelf-life. Low salt meat products are a new class of product and information about their shelf lives and the spoilage process and/ or bacteria implicated in this process haven't been reported. Information available regarding microbiology of cooked ready-to-eat meat products under different storage is scarce and variable due to the fact meat formulation, production process, and handling and storage conditions all tend to differ between studies. Therefore, results obtained under aerobic conditions and vacuum packaging in this study are relevant in order to understand the spoilage process of low salt cooked meat products.

1% NaCl



3% NaCl

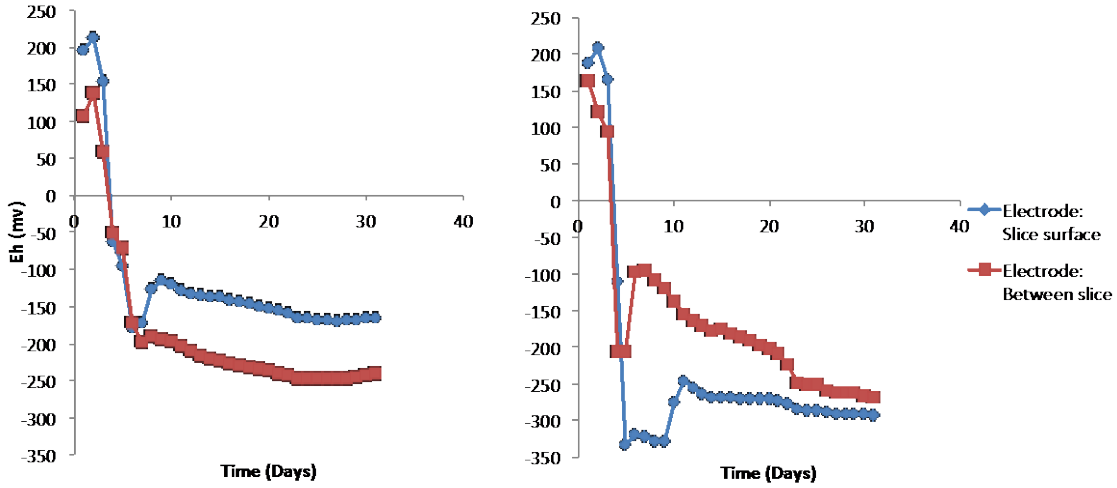


Figure 3.11 Effect of storage and NaCl concentration on redox potential (Eh) measurements of sliced cooked bologna at the slice surface, and between slices, for 30 days on (A) aerobic and (B) vacuum-packaged product on bologna product formulated with 1% and 3% NaCl**.**

Preliminary data exhibited the importance of storage temperature as a key control on spoilage bacteria growth; it was possible to demonstrate that a high temperature result in an large increase in bacterial numbers after only 4 days of storage. When a temperature of 4°C was used,

the significant influence of storage-packaging on microbial growth rate, diversity and microbial shifts during cooked bologna storage was possible to be determined. Overall, the assessment of spoilage bacteria growth under aerobic condition by using culture-dependent techniques demonstrated that LAB, *Br. thermosphacta* and *P. fluorescens* (only in Trial 1) were the dominant microorganisms. Vacuum packaging is known to extend the shelf-life of meat products by retarding bacteria growth and in this study, *P. fluorescens* numbers were lower compared to numbers of LAB and *B. thermosphacta*.

Additionally, redox potential measurements decreased while microbial counts increased, offering an indirect technique as an indicator of microbial growth.

Since research towards microbial ecology of meat products started, culture-dependent techniques have been used to obtain a picture of the diversity of spoilage communities. Overall, this approach has been shown to be unreliable, laborious and costly (Vasilopoulos *et al.,* 2008; Leite *et al.,* 2012). In this study, Ion Torrent high throughput sequence analysis was used to characterize the meta-bacterial community, demonstrating this approach could be applied in order to obtain a more comprehensive description of microbial diversity in this type of food. An assessment of microbial diversity present in low salt cooked bologna during storage and their study under different storage conditions and product formulation may be useful in food microbiological inspections, in the implementation of new packaging systems and in the design of preservation methods (Hu *et al.*, 2009).

3.6. Connection to the next study

Contamination of meat products, especially ready-to-eat (RTE) meats, by pathogenic microorganisms is one of the most important challenges faced by the meat industry since these products don't go through a cooking process after the production stage (Alves *et al*., 2006). In processing plants, contamination of product with foodborne pathogens may occur via direct contact with contaminated processing equipment (Huss *et al*., 2000) or personnel, and their presence not only represents a public health threat, but also an economic loss to the industry due to product recalls. For example, in 2008, a Canadian listeriosis outbreak, linked to ready-to-eat meats produced at a Maple Leaf plant in Ontario, resulted in 20 deaths across five provinces along with 57 total confirmed cases (HC, 2009).

Risk assessments have confirmed *L. monocytogenes* as a common pathogen in sliced and vacuum-packaged cooked meat products (Cornu *et al.*, 2006). This pathogenic bacteria is able to

survive under a wide range of environmental conditions; it is a highly salt-tolerant foodborne pathogen and can multiply under refrigeration temperatures.

Sodium chloride is one of the most frequently used ingredients in meat processing due to its versatility. It contributes to the flavour, texture and microbiological stability of meat products. The aim of reducing sodium content in meat is not a new issue; however, the salt preservative effect may be compromised allowing growth of pathogens such *L. monocytogenes* (Harper and Getty, 2012).

In order for the industry to adopt meaningful reductions in salt content of foods, it is essential that any given product be acceptable regarding quality and safety over its intended entire shelf life. The application of antimicrobial agents such as bacteriocins, (Nisin) and sodium lactate-diacetate has been reported to be an advantageous way to control *L. monocytogenes* in RTE meats (Gill and Holley, 2000; Alves *et al.*, 2006). Accordingly, a second study was carried out aiming to examine the effect of Micocin X® in low salt cooked bologna on the growth of *L. monocytogenes* and spoilage bacteria.

4. SIMULATED POST-PROCESSING CONTAMINATION OF LOW SALT SLICED COOKED BOLOGNA WITH *LISTERIA MONOCYTOGENES*, PRODUCED WITH AND WITHOUT MICOCIN X®

4.1. Abstract

Reducing sodium in food could have an effect on food safety. The main objective of this study was to determine the effect of Micocin X® on the growth of *Listeria monocytogenes* and spoilage bacteria over a 30 day storage period in cooked bologna formulated with l and 3% NaCl under vacuum packaging conditions at 4°C. Cooked bologna, with or without 0.5% (w/w) Micocin X®, was surface inoculated with *Brochothrix thermosphacta*, *Pseudomonas fluorescens*, lactic acid bacteria (*Leuconostoc mesenteroides* and *Lactobacillus curvatus*) and/or *L. monocytogenes.* The bologna was then analyzed over time using culture-dependent and culture-independent techniques to determine the effect of the treatments on microbial growth and community diversity; pH and water activity (a_w) were also measured.

Bacterial counts showed that LAB and *L. monocytogenes* were both affected by bologna containing 3% NaCl, where an increased lag phase of these microorganisms was observed. It was also observed that Micocin X® had a bactericidal effect on *L. monocytogenes*, reducing the viable cell population from 3 log CFU g^{-1} to background values; whereas, no bactericidal effect against the spoilage microflora ($p<0.05$) was seen. These studies demonstrated that *L. monocytogenes* might be controlled in vacuum package cooked bologna formulated with low sodium levels when treated with Micocin X® at a concentration of 0.5% (w/w).

Ion Torrent sequence analysis revealed that the major components of the cooked bologna included the orders *Bacillales* and *Lactobacillales*. Other orders such *Burkholderiales* and *Clostridiales* were also detected. It was further possible to observe increases in the numbers of *Lactobacillales* in the 1% NaCl product formulae over time, whereas an increase of the order *Bacillales* was observed in product formulated with 3% NaCl.

4.2. Introduction

Spoilage bacteria in ready to eat products are generally controlled or eliminated during heat-treatment processing. However, the contamination of vacuum-packaged cooked meat can, and typically does, occur during post-heating, thereby affecting and compromising product safety and quality (Korkeala and Björkroth, 1997; Geornaras *et al*., 2006). Sausages have also been reported to become contaminated during slicing, packaging or during cold storage (Korkeala and Björkroth, 1997).

Listeria monocytogenes is a significant food-borne pathogen frequently associated with ready-to-eat (RTE) products, with contamination occurring during or after processing but before packaging. This microorganism is ubiquitous and has been reported to grow over a wide range of temperature, storage conditions, a_w, pH and sodium concentration (Alves *et al.,* 2006; Martin *et al*., 2010; Harper and Getty, 2012). Therefore, *L. monocytogenes* growth in vacuum-packaged cooked meat presents a significant hazard should post-processing contamination occur.

The United States Department of Agriculture (USDA) has implemented regulations for the industry to prevent contamination and to eliminate *L. monocytogenes* from sensitive food products. The guideline given to processors consists of 3 alternative measures: post-lethality treatment, antimicrobial agents, and sanitation (Martin *et al*., 2010). As an antimicrobial agent, salt is commonly used in the meat industry to inhibit microbial growth, and thus a sodium reduction in salt content has the potential to compromise food safety (Harper and Getty, 2012). Hurdle technology has proven to be an effective approach for ensuring the microbiological safety of food. Hurdle technology is based on the combination of different, sub-lethal methods of preservation or techniques (hurdles) in order to achieve an equivalent result as where a single preservation technique (e.g., thermal treatment) is applied at a lethal level. The most common hurdles used in meat industry include physical heat, pH, and water activity. As mentioned previously, a reduction in sodium concentration would affect the water activity of the food, thereby necessitating the implementation of an alternate hurdle.

Bacteriocins have been extensively studied due to their potential use as an element of hurdle technology. Micocin X® is a commercially-available dried preparation of *C. maltaromaticum* UAL307 cell-free culture that contains carnocyclin A, piscicolin 126 and carnobacteriocin BM1, and has recently been approved by Health Canada as a biopreservative. However, the effect of Micocin X® on *L. monocytogenes* has not yet been extensively studied.

Based on the importance of sodium reduction and the potential for bacteriocins to offer prolonged control of serious pathogens of concern to the meat industry, the main objective of this study was to evaluate the inhibition of *L. monocytogenes* and spoilage microorganisms during extended storage at 4°C by the addition of Micocin X® in vacuum-packaged cooked meat bologna formulated at low (1%) and high (3%) NaCl levels. Supplementation of product formulae with this bacteriocin is postulated to offer additional antimicrobial activity, particularly against *L. monocytogenes,* resulting in an extension of meat quality and shelf life compared to that of controls.

4.3. Materials and Methods

4.3.1. Experimental design

The effects of Micocin X® and sodium concentration (1 and 3% NaCl) on the growth of *Listeria monocytogenes, Pseudomonas fluorescens, Brochothrix thermosphacta* and LAB was evaluated on vacuum-packaged sliced cooked bologna stored at 4°C over 30 days. Sampling was performed every third day over the first 15 days, and then every fifth day, thereafter. Pork bologna was formulated with, and without Micocin X®, and each treatment was incubated under inoculated and uninoculated conditions as shown in Figure 4.1.

Over the course of Study 2, a second biological replicate (Trial 2) was carried out after completion of the first trial. However, after running the statistical analysis it was determined that there was excessive variation between trials regarding the growth of *L. monocytogenes;* thus, the analysis for this microorganism was not combined (Fig 4.2).

4.3.2. Manufacture of cooked bologna

Fresh pork meat was obtained from a commercial meat processor through a local meat supplier. Bologna was formulated to target a fat content of 20.0% and a protein content of 11.0%, as in the first study. The level of water and salt varied according to the sodium concentration treatment, as shown in Table 4.1.

Micocin X® (Griffith Laboratories; Toronto, ON) was added into the bologna blend with non-meat products. Bologna processing was carried out as described in section 3.3.1.

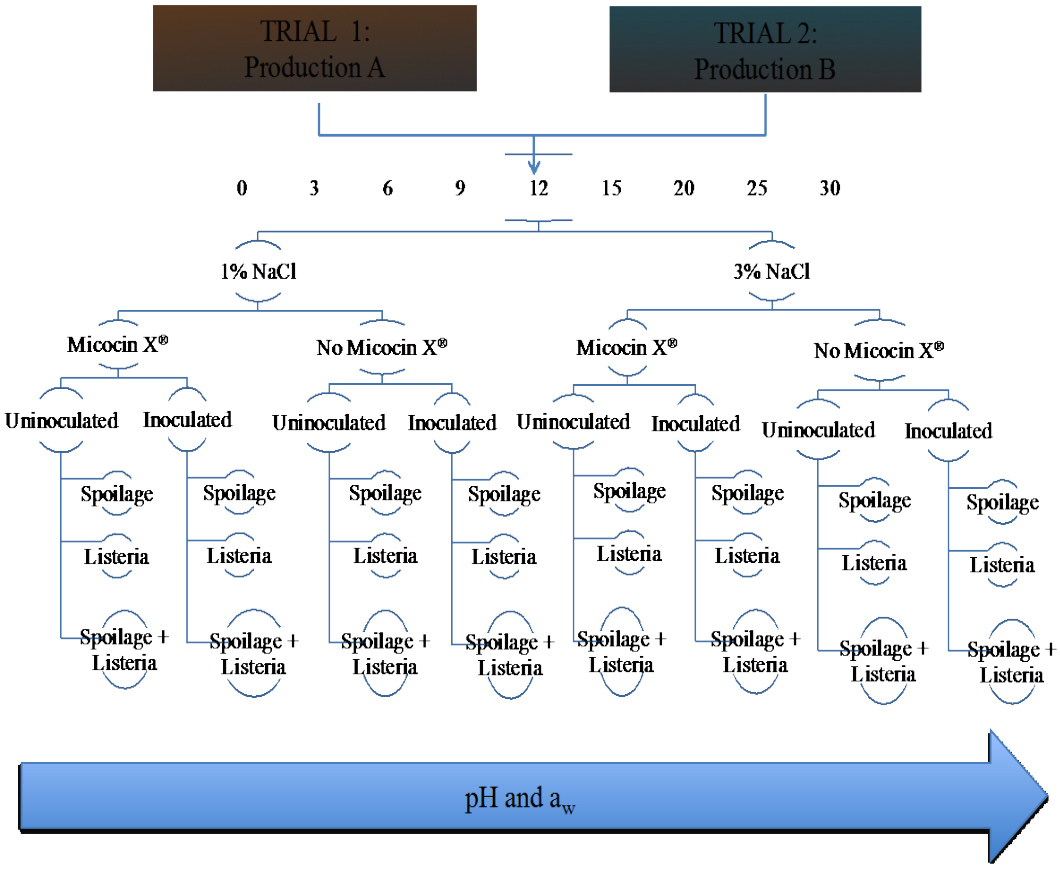


Figure 4.1 Schematic diagram illustrating the experimental procedure conducted over 30 days during Trial 1 and Trial 2 at a temperature of 4°C. Each trial utilized cooked bologna formulated with 2 different (1 and 3% NaCl) sodium concentrations, with or without Micocin X®, which were then stored under vacuum-packaged conditions. Each treatment included uninoculated control samples as well as meat samples inoculated with spoilage bacteria. pH and a_w measurements were made at intervals indicated in the diagram timeline.

4.3.3. Bacteria and preparation of inoculum

Listeria monocytogenes was obtained from the Microbiology Laboratory Culture Collection at University of Saskatchewan. To prepare the inoculum, cultures inoculated from frozen stock were grown overnight in 100 mL of Brain-Heart Infusion broth (BHI, Becton Dickinson, Franklin Lakes, NJ, USA), after which 1 mL was transferred to a fresh flask, followed by an additional 24 hr incubation at 37°C. The cells were then harvested by centrifugation (10000 x g for 10 min), washed twice and resuspended in 0.1% peptone water. Spoilage bacteria and co-culture procedures are the same as described in section 3.3.4.

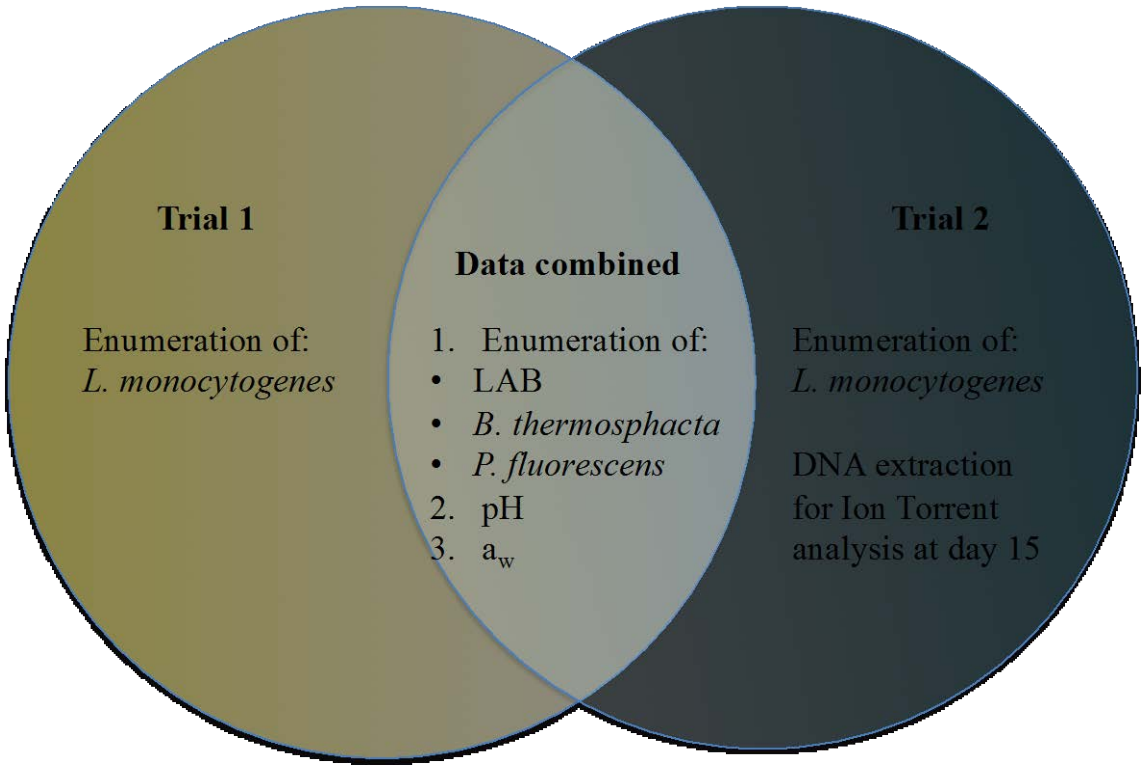


Figure 4.2 Diagram illustrating data analysis. The overlapping area indicates which data obtained over the 30 days study period was combined. Ion torrent analyses were conducted from samples obtained in Trial 2.

4.3.4. Inoculation procedure

Refer to section 3.3.5 for procedural details.

4.3.5. Microbial analysis

The protocol for enumerating different bacteria is as previously described in section 3.3.6. Counts of *L. monocytogenes* were determined using medium containing polymyxin B, acriflavin, lithium chloride, ceftazidine, esculin, and mannitol (PALCAM; Difco Laboratories Inc., Detroit, MI).

Table 4.1 Bologna formulation (%, w/w) with two different NaCl levels, with and without Micocin X®

Ingredients	1%	1%	3%	3%
Pork leg muscle, %	55.83	55.83	55.83	55.83
Pork backfat, %	19.17	19.17	19.17	19.17
Ice water, %	20.95	20.45	18.95	18.45
NaCl, %	0.71	0.71	2.71	2.71
Prague powder, %	0.30	0.30	0.30	0.30
Sodium erythorbate, %	0.05	0.05	0.05	0.05
Sodium tripolyphosphate, %	0.40	0.40	0.40	0.40
German wiener seasoning, %	0.60	0.60	0.60	0.60
Wheat flour, %	2.00	2.00	2.00	2.00
Micocin X®, %	-	0.50	-	0.50

4.3.6. Water activity (A_w)

Refer to section 3.3.8 for procedural details.

4.3.7. pH measurements.

Refer to section 3.3.9 for procedural details.

4.3.8. DNA extraction and PCR amplification

Refer to section 3.3.11 for procedural details.

4.3.9. Ion Torrent 16S rRNA gene sequencing

The procedural details for Ion Torrent 16S rRNA gene sequencing were previously explained in section 3.3.12. The MIDs used for each meat treatment were as follows: CATAG

(1% NaCl inoculated with spoilage microflora), CGAGA (1% NaCl inoculated with spoilage microflora + *Listeria*), ATACG (1% NaCl inoculated with *Listeria*), TCACG (1% NaCl + Micocin X®, inoculated with spoilage microflora), CGTCT (1% NaCl + Micocin X®, inoculated with spoilage microflora + *Listeria*), TCTAC (1% NaCl + Micocin X®, inoculated with *Listeria*), TGTAC (3% NaCl inoculated with spoilage microflora), CGTAG (3% NaCl inoculated with spoilage microflora + *Listeria*), TACGA (3% NaCl inoculated with *Listeria*), TACTC (3% NaCl + Micocin X®, inoculated with spoilage microflora), CGACG (3% NaCl + Micocin X®, inoculated with spoilage microflora + *Listeria*), AGTAC (3% NaCl + Micocin X®, inoculated with *Listeria*).

4.3.10. Statistical analysis

Refer to section 3.3.13 for procedural details. Microbiological data obtained over the 30 day storage period was grouped and analyzed as shown in Figure 4.2.

4.4. Results and Discussion

4.4.1. Enumeration of LAB

Lactic acid bacteria initial counts on inoculated samples were approximately 3.5 log CFU g^{-1}. Numbers increased significantly throughout the storage time course ($P < 0.05$) to around 8 log CFU g^{-1} over the first 9 days on bologna formulated with 1% NaCl and Micocin X® (Figure 4.3A).

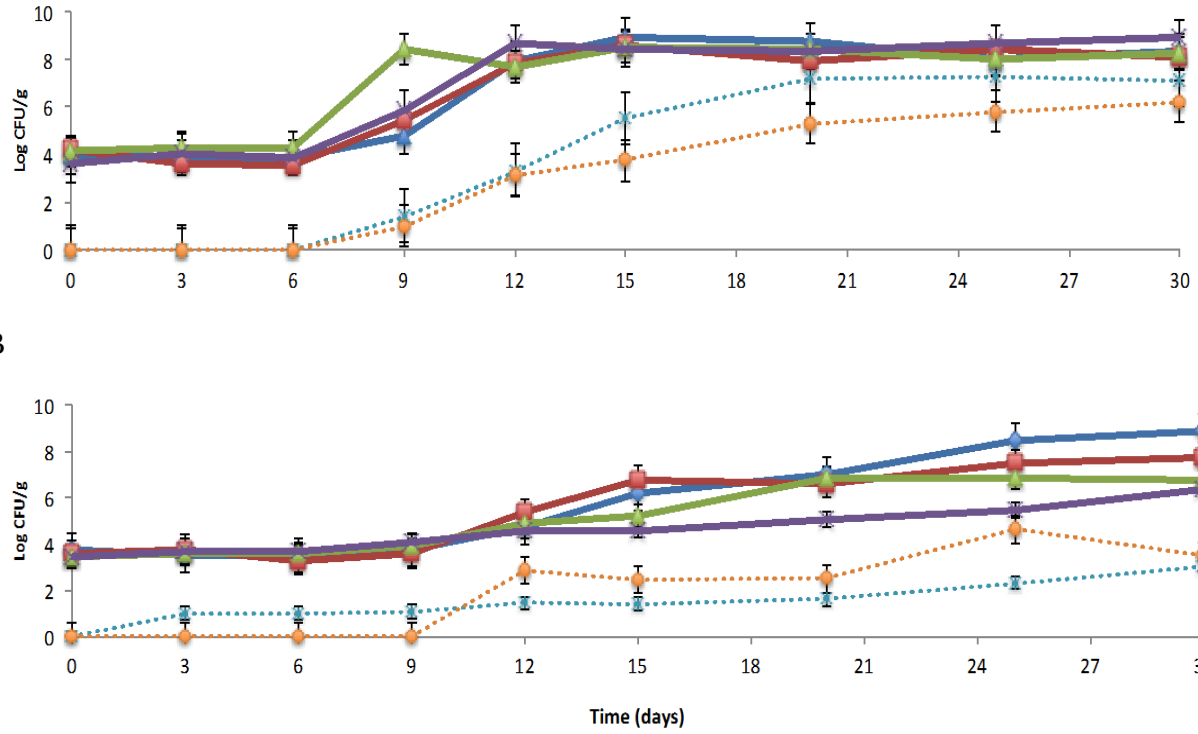
As expected (and from the results of the first study), the highest salt concentration (3% NaCl) (Figure 4.2B) affected bacteria growth ($p<0.05$), as reflected by a longer lag phase. When LAB growth was assessed in the bologna formulated with 3% NaCl, with and without Micocin X®, no significant differences were found ($P > 0.05$). These results are not surprising since bacteriocins are very specific and do not broadly inhibit all spoilage microorganisms (Korkeala and Björkroth, 1997). Notably, bacteriocin producers are resistant to their own bacteriocin; nonetheless, this insensitivity has also been observed in non-bacteriocin producing cells (Nes and Holo, 2000; Eijsink *et al*., 2002).

It is known that this general resistance of non-bacteriocin producer cell towards bacteriocins is due to the expression of immunity genes (Kjos *et al*., 2011a). Lactic acid bacteria have been reported to possess immunity genes that vary in terms of expression levels and that

provide different degrees of protection towards different class of bacteriocins (Eijsink *et al.*, 2002).

Proteins that confer bacteriocin immunity are often located within the same operon as the bacteriocin structural gene(s) like the immunity protein EntqC and LagC of the class IIb bacteriocin lactococcin G (Kjos *et al.*, 2011a). The mechanism by which they work is by kidnapping the structural protein or by interacting with a putative receptor (Balciunas *et al.*, 2013). Protection has also been reported to be provided by specialized ABC transporters, like in the case of enterocin AS-48, where the transporters pump bacteriocins out from the cytoplasmatic membrane into the extracellular medium (Diaz *et al.*, 2003; Balciunas *et al.*, 2013).

A



B

Figure 4.3 A, B Numbers of viable LAB on sliced cooked, vacuum-packaged bologna formulated with or without Micocin X® and with 1% NaCl (A) or 3% NaCl (B) without Micocin X® (♦), in the presence of *Listeria* and without Micocin X®(■), alone with Micocin X® (▲), in the presence of *Listeria* and Micocin X® (×) uninoculated without Micocin X® (--*--), uninoculated with Micocin X® (--●--) stored at 4°C.

Table 4.2 *P*-value for estimated parameters for LAB growth in combination with *L. monocytogenes* in vacuum-packaged sliced cooked bologna

Effect	LAB+ Listeria
Salt	0.0024
Micocin X®	0.6073
Background	0.1387
Day	<.0001
Salt* Micocin X®	0.3453
Micocin X® *Background	0.5297

4.4.2. Enumeration of *Brochothrix thermosphacta*

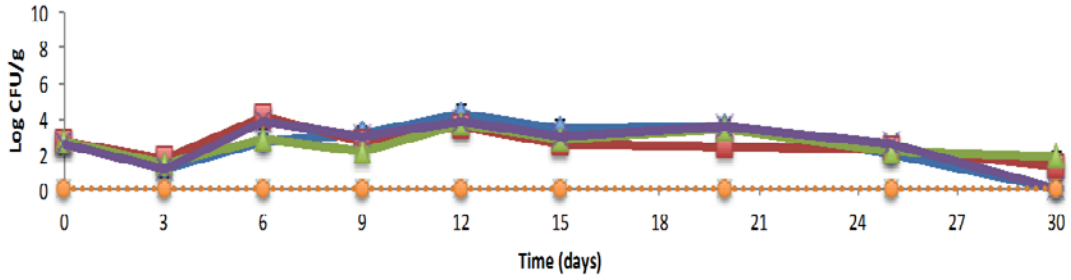
Brochothrix thermosphacta numbers obtained during this study on bologna formulated with 1% NaCl are shown in Figure 4.4A. As shown in Table 4.3, it was possible to determine there was no Micocin X® effect on the growth of this spoilage microorganism (P >0.05) and also, as in study 1 (Chapter 3), it was possible to determine there was not a salt effect on microbial growth. Regarding the presence of Listeria, data suggested that under the conditions used in this study, there was no effect on *B. thermosphacta*.

In a study conducted by Martin-Visscher *et al.* (2008), purified carnocyclin A was tested against *B. thermosphacta* ATCC 11509 using the spot-on-lawn assay technique in soft agar, and in this experiment an inhibition of this Gram-positive bacteria was assessed. However, this discrepancy in results can be supported by the fact that bacteriocin activity has been reported to be affected by the food matrix (Campos *et al.*, 2013).

Table 4.3 *P*-value for estimated parameters for *Brochothrix thermosphacta* growth in absence and presence of *L. monocytogenes* in vacuum-packaged sliced cooked bologna

Effect	*B. thermosphacta*	*B. thermosphacta* + Listeria
Salt	0.5457	0.6223
Micocin X®	0.8548	0.8716
Day	0.2063	0.0437
Salt* Micocin X®	0.879	0.8485

A



B

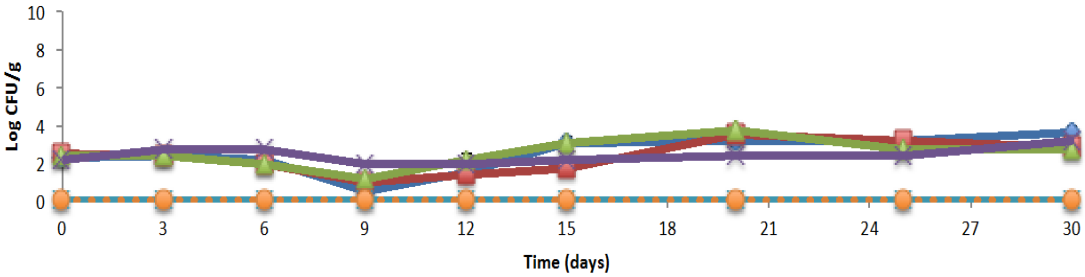


Figure 4.4 A, B Numbers of viable *Brochothrix thermosphacta* cells on sliced cooked, vacuum-packaged bologna formulated with or without Micocin X® and with 1% NaCl (A) or 3% NaCl (B) without Micocin X® (♦), in the presence of *Listeria* and without Micocin X®(■), with Micocin X® (▲), in the presence of *Listeria* and Micocin X® (×) uninoculated without Micocin X® (--*--), uninoculated with Micocin X® (--●--) stored at 4°C.

4.4.3. Enumeration of *Pseudomonas fluorescens*

The numbers of *P. fluorescens* cells recovered from high (3% NaCl) and low (1% NaCl) salt vacuum-packaged bologna formulated with or without Micocin X® are shown in Figure 4.5. The results demonstrated that microbial growth was significantly ($p<0.05$) affected by sodium concentration as well as the presence of Micocin X® (table 4.4). It was possible to determine there was an interaction between Micocin X® and salt concentration; bacteria numbers obtained on 1% NaCl in the presence of Micocin X® were not significantly different from those obtained in 3% NaCl in the presence or absence of bacteriocin as in shown in Table 4.5.

Table 4.4 *P*-value for estimated parameters for *Pseudomonas fluorescens* growth in absence and presence of *L. monocytogenes* in vacuum-packaged sliced cooked bologna

Effect	*P. fluorescens*	*P. fluorescens* +Listeria
Salt	0.0054	0.0098
Micocin X®	0.0047	0.0076
Day	0.0081	0.0743
Salt* Micocin X®	0.0918	0.0172

In this study, in the absence of Micocin X® at a sodium concentration of 1% NaCl, *P. fluorescens* increased in numbers from 2.69 to 3.08 log CFU g^{-1} at the end of the storage period; whereas on 3% NaCl, a reduction in bacteria numbers from 2.54 log CFU g^{-1} to the minimum detection limit was assessed by day 25.

Table 4.5 Differences of least square means for growth of *Pseudomonas fluorescens* at 1 and 3% NaCl in the presence and absence of Micocin X®.

Sodium % (w/w)	Micocin X®	Least Squares Means
1	-	2.8612^{A}
3	-	1.5783^{B}
1	+	1.5434^{B}
3	+	0.9644^{B}

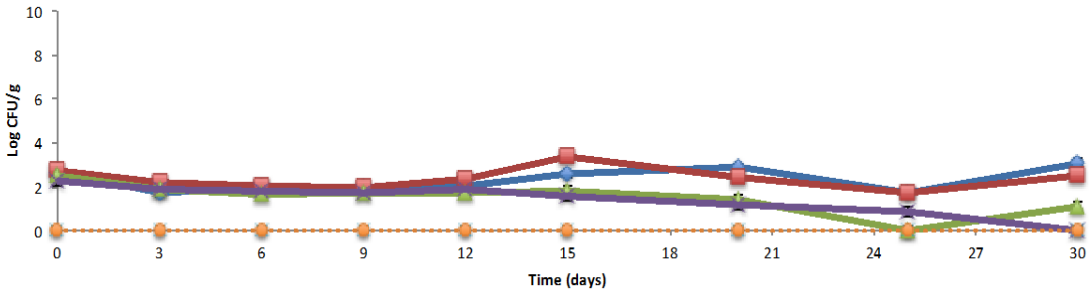
*Means with the same letter are not significantly different ($p < 0.05$)

When comparing results obtained from treatments with and without Micocin X®, it was possible to determine that on 1% NaCl treatment, the addition of Micocin X® to the meat formulae resulted in a reduction of ~ 0.8 log CFU g^{-1} by day 15, decreasing from 2.60 to 1.78 log CFU g^{-1} and approximately 2 log CFU g^{-1} reduction by the end of the study. On 3% NaCl, the presence of Micocin X® resulted in a reduction of ~ 0.9 log CFU g^{-1} when compared to the treatment without bacteriocin; numbers decreased from approximately 1.5 log CFU g^{-1} to 0.5 log CFU g^{-1} by day 15.

The presence of *Listeria* did not affect *P. fluorescens* and bacteria numbers were maintained between 2.74 and 1.71 log CFU g^{-1} through the study period; whereas on 3% NaCl, a reduction from 2.29 log CFU g^{-1} to the minimum detection limit was assessed at day 25 and thereafter until the end of the study. On the other hand, on 1% NaCl in the presence of Listeria

and Micocin X®, numbers of *P. fluorescens* decreased from 2.25 log CFU g^{-1} to 0.85 log CFU g^{-1}; whereas a reduction from 2.28 log CFU g^{-1} to the minimum detection limit was assessed by day 20 until the end of the study on 3% NaCl treatment.

A



B

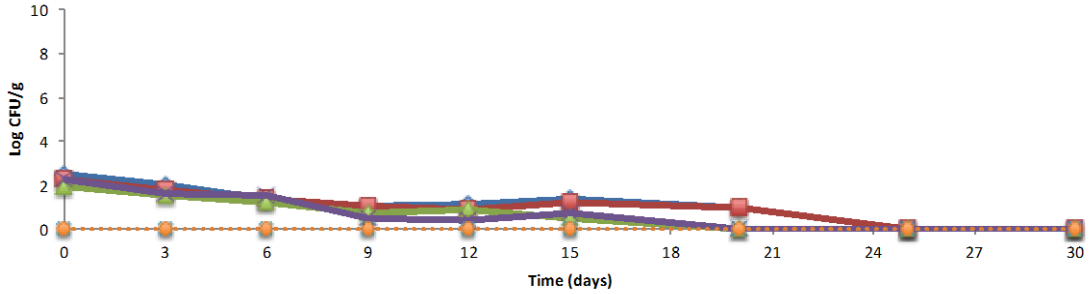


Figure 4.5 A, B Numbers of viable *Pseudomonas fluorescens* cells on sliced cooked, vacuum-packaged bologna formulated with or without Micocin X® and with 1% NaCl (A) or 3% NaCl (B) without Micocin X® (♦), in the presence of Listeria and without Micocin X®(■), with Micocin X® (▲), in the presence of Listeria and Micocin X® (×) uninoculated without Micocin X® (--*--), uninoculated with Micocin X® (--●--) stored at 4°C.

4.4.4. Enumeration of *Listeria monocytogenes*

Several reports have demonstrated the antagonistic effect of bacteriocins towards *L. monocytogenes* in cooked meat products (Alves *et al.*, 2006; Castellano and Vignolo, 2006). The hypothesis of this study was that as sodium was reduced in the formulation, *L. monocytogenes* would proliferate more rapidly and that the presence of the bacteriocin would inhibit bacterial growth.

As expected (from Trials 1 and 2), more rapid growth of *L. monocytogenes* was observed on meat formulated with 1% NaCl in comparison to the 3% NaCl treatment. When meat formulated with 1% NaCl was evaluated (Figure 4.6), it was noted that the bologna formulations without Micocin X®, or in the absence of spoilage microorganisms, *L. monocytogenes* attained 5.4 log CFU g^{-1} after 12 and 20 days of storage and a maximum cell density of 7.6 log CFU g^{-1} by the end of both trials. Again, this result was expected, since no antimicrobial treatment or hurdle technology was applied to inhibit or reduce the growth of this pathogen.

The growth of *L. monocytogenes* was significantly affected by the presence of spoilage bacteria in meat in the absence of Micocin X® ($p<0.05$), suggesting that competition for nutrients had occurred (Alves *et al*., 2006). In fact, the influence that different spoilage microorganisms have on the growth of *L. monocytogenes* is well documented (Buchanan and Bagi, 1999; Marshal *et al.,* 1992; Tsigarida *et al*., 2000). For example, LAB are widely-known to compete with, and inhibit, *L. monocytogenes* within a food system through the production of both organic acid and bacteriocins. Lactic acid bacteria compete by lowering pH of the medium to levels unfavourable for *Listeria* and other pathogens.

The ability of *Brochothrix* spp. to affect *L. monocytogenes* has been less studied than LAB. However, *Brochothrix* spp. have been reported to produce, under anaerobic conditions, lactate, acetate, ethanol, bacteriocins, as well as short chain fatty acids. A variety of these metabolites are also known to be produced by LAB (Cayré, 2005; Grau, 1980), for instance, it is expected that this microorganism affects the growth/survival of *L. monocytogenes*.

In Trial 1, in the absence of spoilage bacteria, there was a Micocin X® effect and interaction between Micocin X® and salt on the growth of *L. monocytogenes.* When *L. monocytogenes* was grown on meat formulated with 1% salt and Micocin X®, a large, significant effect was noted when compared to the treatment without bacteriocin, with an ~6.5 log CFU g^{-1} difference detected at the end of the study (Figure 4.6A). When *L. monocytogenes* was inoculated on meat without Micocin X® and spoilage bacteria on 1% salt, *L. monocytogenes* numbers increased from 2.68 log CFU g^{-1} to 4.44 log CFU g^{-1} by the end of the study; whereas the presence of Micocin X® and spoilage bacteria growth decreased from 2.68 log CFU g^{-1} to the detection limit by day 25.

In bologna product formulated with 3% NaCl during Trials 1 and 2, a longer lag phase was detected in all treatments relative to the 1% NaCl-formulated product, in agreement with

results found by Zarei *et al.* (2012) who reported that the lag phase of *L. monocytogenes* increased as the sodium concentration was increased from 1 to 9 % NaCl. In these two high-salt trials, the number of *L. monocytogenes* on meat without bacteriocin or spoilage bacteria gradually increased, reaching a maximum number around 4.8 log CFU g^{-1} by the end of the study (Figure 4.6AB).

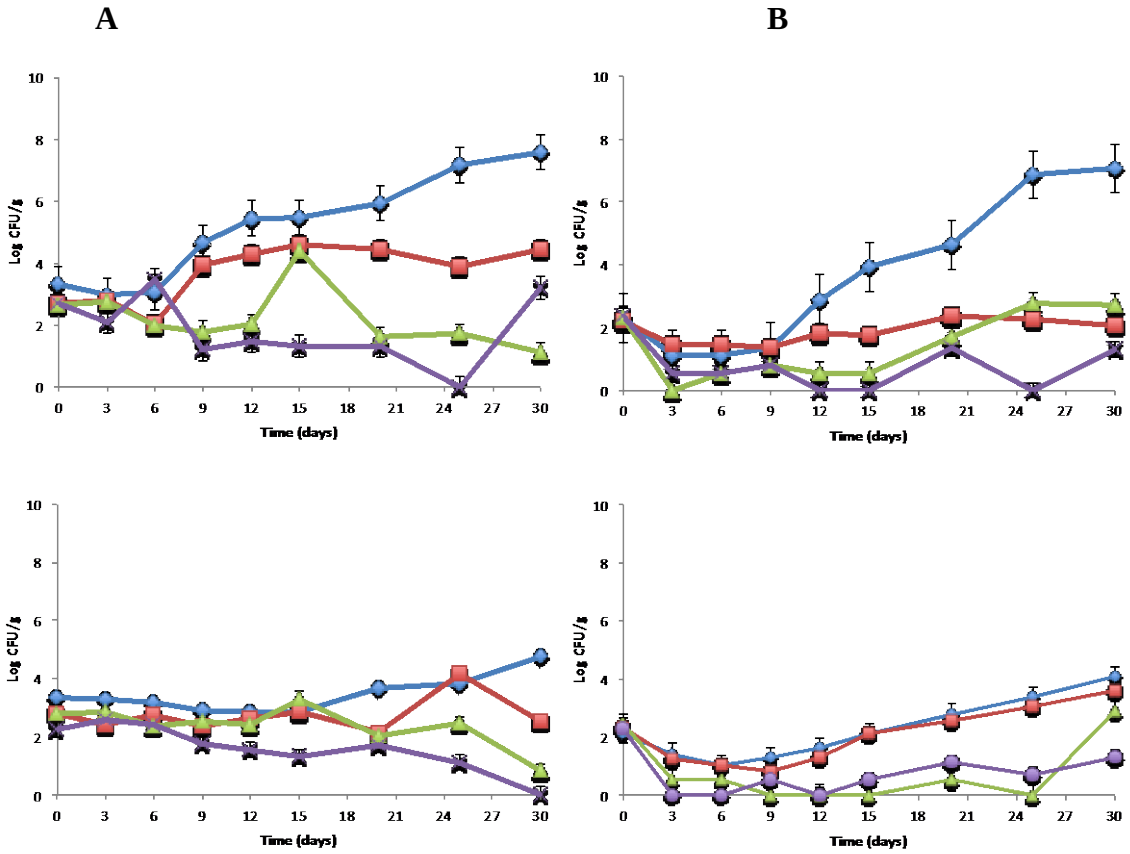


Figure 4.6 Numbers of viable *Listeria monocytogenes* on sliced cooked, vacuum-packaged bologna during (A) Trial 1 and (B) Trial 2. On meat formulated without Micocin X® (♦), in the presence of spoilage bacteria and without Micocin X®(■), with Micocin X® (▲), in the presence of spoilage bacteria and Micocin X® (●) stored at 4°C.

These increases in numbers at the end of the experiment suggest there is most probably an adaptation of *L. monocytogenes* to Micocin X®. In a recent study conducted by Liu *et al* (2014), it was demonstrated and adaptation of *Listeria* to a sublethal dose of carnocyclin A (cclA). After 30 h exposure to cclA it was demonstrated there is a down-regulation of the mannose-specific and cellobiose-specific PTS systems; a mechanism that has been observed in both spontaneous

resistant mutants and in natural resistant isolates (kjos *et al.*, 2001b; Liu *et al.*, 2014).

Listeria monocytogenes is ubiquitous and it is able to sustain growth at a minimum water activity of 0.90 and an optimal pH value of 7.0. During the preliminary study, it was observed that sodium concentration didn't have a discernable effect on *L. monocytogenes* growth. However, in Trial 2, it appeared that *L. monocytogenes* was significantly affected by sodium concentration (Figure 4.6; Table 4.6). The combined effect of water activity, indigenous spoilage bacteria and pH seem to have further affected the susceptibility of this pathogen to sodium concentration.

Even though results from both trials were statistically different, it was observed that in both cases there was a large reduction in *L. monocytogenes* numbers on meat formulated with Micocin X® and on meat co-inoculated with spoilage bacteria formulated with Micocin X®. In both studies, at some time intervals, the number of *L. monocytogenes* dropped below the detection limit of the plate count method. Accordingly, enrichment for *Listeria monocytogenes*, followed by presence or absence confirmatory testing, was performed in accordance with the FDA protocol and demonstrated that viable *L. monocytogenes* cells were still present.

Table 4.6 *P*-value for estimated parameters for *Listeria monocytogenes* growth in absence and presence of spoilage bacteria in vacuum-packaged sliced cooked bologna.

Effect	Trial 1		Trial 2	
	Listeria	**Listeria + spoilage**	**Listeria**	**Listeria + spoilage**
Salt	0.0638	0.2172	<.0001	0.0714
Micocin X®	<.0001	0.0564	<.0001	0.0248
Day	0.7746	0.9996	1	0.0071
Salt* Micocin X®	0.025	0.6251	<.0001	0.1856

4.4.5. Chemical analyses

The physical chemical parameters of the bologna meat product were basically unaffected by the different treatments and salt concentration as expected. Water activity remained constant (0.99-0.98) on uninoculated meat product containing 1% NaCl with and without Micocin X® over the course of the study. On uninoculated 3% NaCl meat product, the water activity remained constant between 0.96-0.97. The pH values were observed to drop in conjunction with microbial

growth. In bologna product containing 1% salt, the pH decreased from 6.5 to 5.8 where spoilage bacteria were added and underwent a slight decreased to 6.3 on meat just inoculated with *L. monocytogenes*. In 3% NaCl meat product, the pH changed marginally from 6.5 to 6.3 on meat inoculated with spoilage bacteria and on meat inoculated with *L. monocytogenes*. In meat formulated with Micocin X® pH values were not significantly different from meat formulated without it.

4.4.6. Bacterial community determined by culture-independent technique

In the present study a total of 97,448 16S rRNA sequence reads were obtained and the number of sequences for each samples are presented in Table 4.7.

Table 4.7 Number of sequences observed and analyzed for 16s rRNA of bologna samples formulated with two sodium concentrations with or without bacteriocin.

% NaCl (w/w)	Sample	Read number
1	Spoilage	3037
	Micocin X® + Spoilage	12718
	Spoilage + Listeria	2442
	Micocin X® + Spoilage + Listeria	14615
	Listeria	1707
	Micocin X® + Listeria	11250
3	Spoilage	14956
	Micocin X® + Spoilage	9779
	Spoilage + Listeria	3618
	Micocin X® + Spoilage + Listeria	4373
	Listeria	14507
	Micocin X® + Listeria	4446

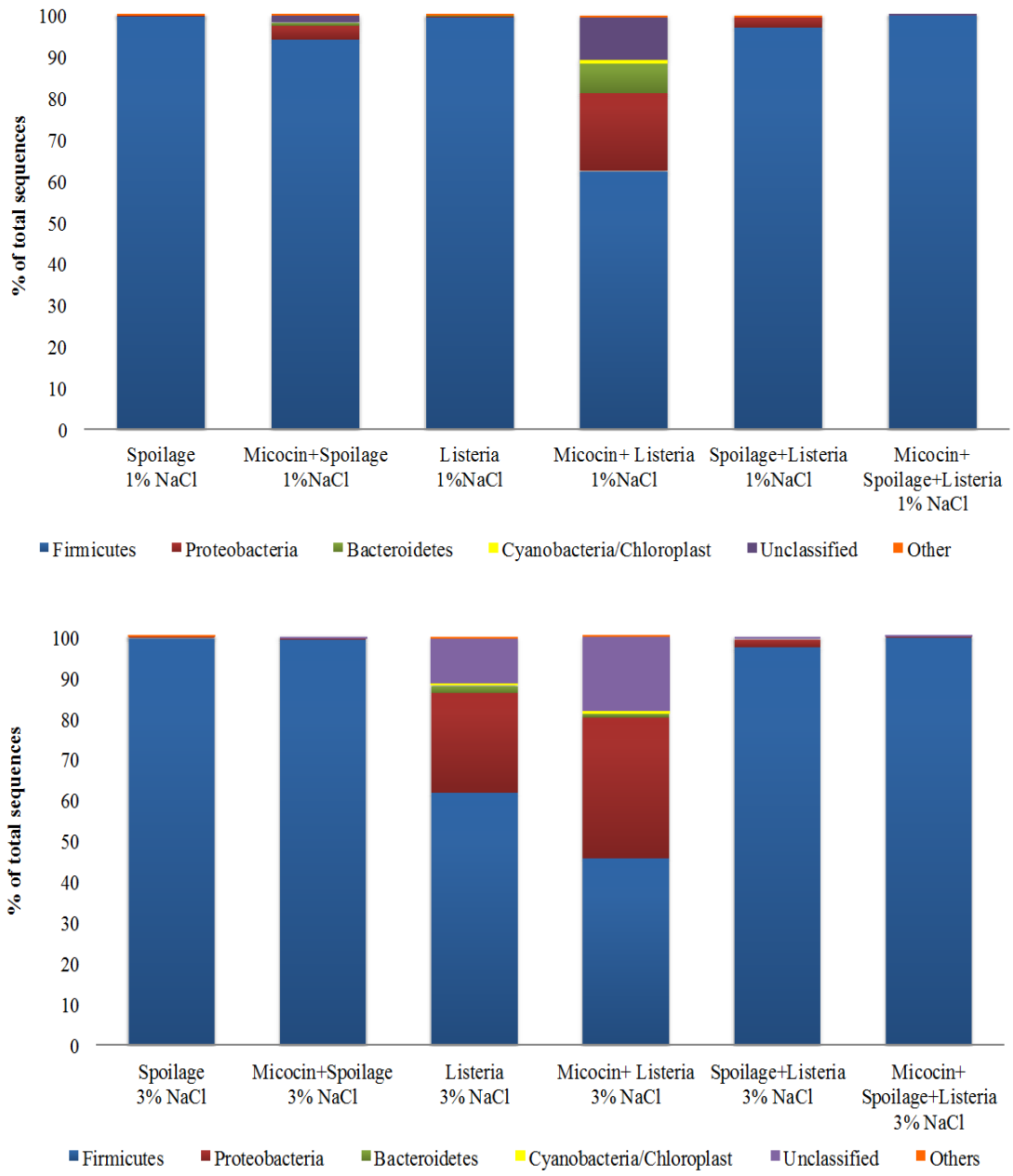


Figure 4.7 Taxonomic classification of bacterial reads at phylum level using RDP classifier with a confidence threshold of 50% retrieved from inoculated and uninoculated sliced cooked bologna formulated with 1% (Top), and 3% (Bottom) NaCl, in the presence or absence of Micocin X®, stored under aerobic and vacuum conditions at 4°C, sampled at day 15 from Trial 2.

All bologna samples were dominated by the phylum *Firmicutes*, with a relative abundance of 92.1% on 1% NaCl meat formulations and 84% on the 3% NaCl formulae. The second most abundant group was *Proteobacteria,* but its relative abundance varied significantly within samples, ranging from 0.2 to 18.8% on 1% NaCl formulae and between 0.2 and 34.5% on 3% NaCl formulae. Finally, a minor percentage, between 0.8 and 7.3% of *Bacteroides* and unclassified bacteria (0.1%) were also detected.

Bacterial counts combined with microbial identification data allowed the study of the impact of storage condition and the effect of bacteriocin on bacteria growth. As shown in Figure 4.8, five major orders were detected on meat formulated with 1% NaCl. However, some variations of the represented orders were observed probably due to variations in DNA concentrations.

It is well established that strains of LAB are the major group of spoilage bacteria on vacuum-packed meat and meat products (Bjorkroth & Korkeala, 1997; Hu *et al.*, 2009), and as it was determined on MRS agar (section 4.2.1), LAB numbers increased regardless of the presence or absence of Micocin X®. In this study, on meat product containing 1% NaCl, an increase of *Lactobacillales* was observed, more specifically, microorganism belonging to the family *Lactobacillaceae*, *Leuconostocaceae* and *Carnobacteriaceae*. The inhibitory effect of carnocyclin A against *B. thermosphacta*, *C. mataromaticum*, *Leuconostoc mesenteroides, Lactobacillus, Enterococcus faecalis and E. faecium* has been reported in an earlier study conducted by Martin-Visscher *et al.* (2008). However, it has been reported that food matrices may negatively affect bacteriocin activity and in this study, the interaction between the food matrix and the bacteriocin was not evaluated.

The effectiveness of bacteriocins activity in food depends on many factors such retention of the bacteriocins molecules by food system components like fat and emulsifiers or due to the interaction with food matrix (Galvez *et al*., 2007; Campos *et al.*, 2013). In sausages, it has been found that in products with low fat contents there is a higher nisin activity. With respect to the interaction with the food matrix, it has been reported that nisin is inactivated by glutathione, which is found in raw meat (Campos *et al.*, 2013). Finally other conditions that may affect bacteriocins activity are inactivation by other additives, slower diffusion and solubility and/or uneven distribution of bacteriocins molecules in the meat matrix (Galvez *et al*., 2007; Campos *et al.*, 2013).

When comparing the percentage of sequences of *Lactobacillales* and *Bacillales* obtained from 1% and 3% NaCl treatment (Figure 4.8), it was possible to observe high frequencies of the order *Bacillales* at 3% NaCl. As it has been mentioned before, LAB are more susceptible to high sodium concentrations; for instance, it was expected that the order *Bacillales*, represented by the genus *Brochothrix*, would dominate the system on meat formulated with 3% NaCl, whereas *Lactobacillales* would dominate the meat system on 1% NaCl formulae.

Despite the fact that *Proteobacteria* community is also a major group, it was not detected in all samples at the same abundance. The sequences of this phylum on 1% and 3% NaCl belonged to the class *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaprotobacteria* and were represented by the genus *Pseudomonas, Burkholderia, Acidovorax, Xanthomonas, Acinetobacter, Shewanella*, *Sphingobium and Bradyrhizobium*. These microorganisms are typically found in soil and water and are widely distributed in fresh foods, such as meats and poultry, and only a relatively few are able to establish their presence on or in the plant environment (Jay *et al*., 2004).

Since most *Proteobacteria* associated with meat processing have been recovered from meat contact surfaces, their presence in cooked meat product indicates that post-processing contamination with contact surfaces had likely occurred. For instance, to avoid contamination by potential spoilage bacteria, it is recommended to have separate rooms for cooked products and raw meat, more control on material flow and special decontamination procedures of floors and equipment such as knives and cutting boards (Korkeala and Björkroth, 1997; Welker *et al*., 1997).

Although there are several studies that have revealed the microbial dynamics in different foods, this is the first study that aimed to reveal microbial composition of cooked bologna using high throughput sequence analysis. In general, this technique allowed a better description of the bacteria present in cooked bologna under two different sodium concentrations in the presence and absence of Micocin X®. However, the proportion of unclassified microbial groups (sequences that belong to the Bacteria root but were unable to identify precise taxonomy) increased as the rank of classification increased, probably due to the short length of the sequences (100 bp) or due to the low DNA concentration, affecting the detection at a more specific level.

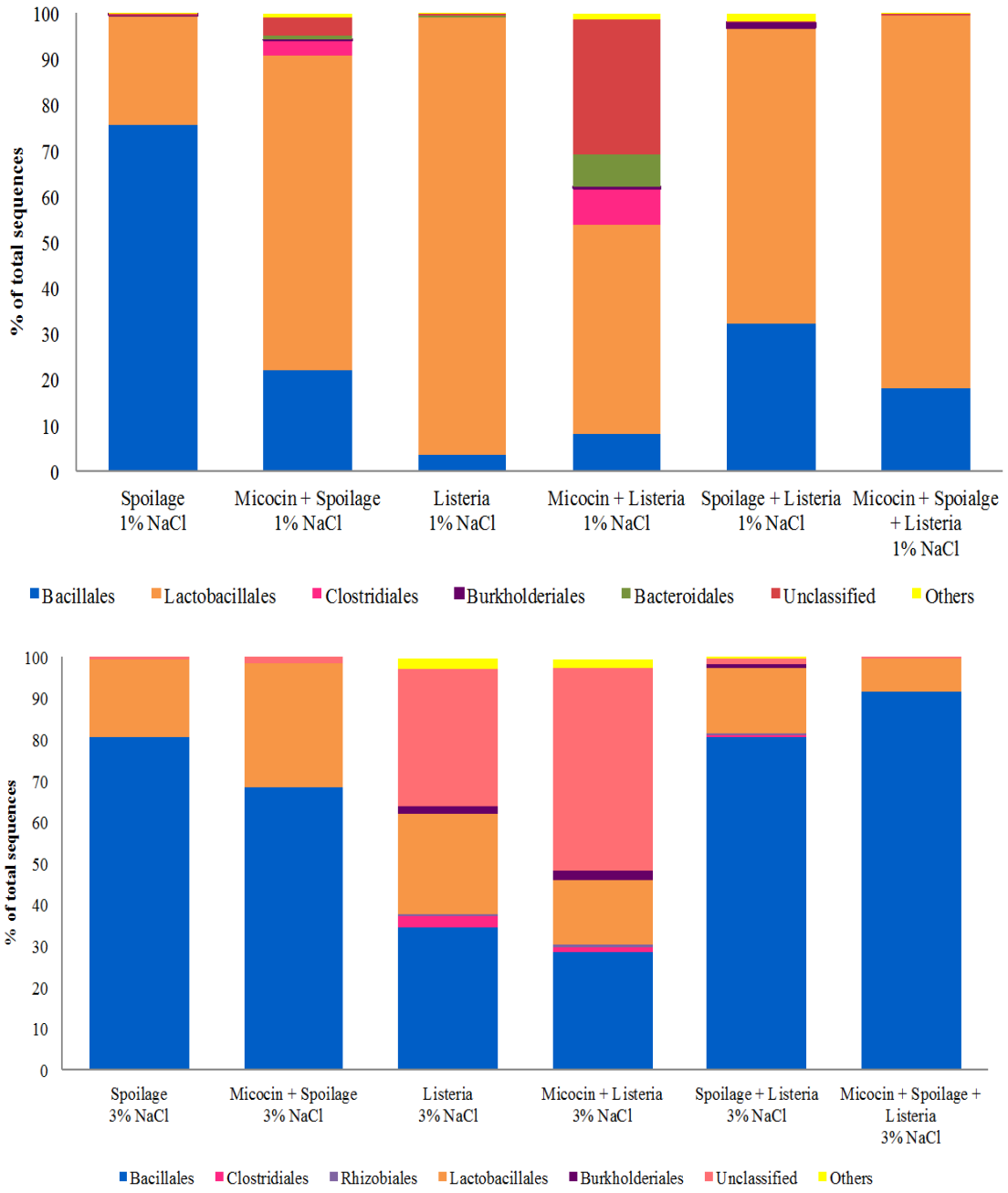


Figure 4.8 Taxonomic classification of bacterial reads at order level using RDP classifier with a confidence threshold of 50%, retrieved from inoculated and uninoculated cooked bologna formulated with 1% (Top), and 3% (Bottom) NaCl, in the presence or absence of Micocin X®, stored under aerobic and vacuum conditions at 4°C, sampled at day 15 from Trial 2.

4.5. Conclusions

It is well understood that the sodium reduction strategy on meat products is pushing the industry to come with new hurdle technologies that will control and inhibit the growth of *L. monocytogenes* in ready-to-eat (RTE) products. This study proposed the incorporation of Micocin X® into the meat blend to control the incidence of *L. monocytogenes* on sliced cooked bologna vacuum-packaged under storage conditions (4°C).

Based on the results obtained in this study, it was possible to conclude that Micocin X® has a significant effect on *P. fluorescens* and *L. monocytogenes;* whereas, there was no significant effect on growth of LAB and *B. thermosphacta.* The study further revealed that *L. monocytogenes* could be controlled by a Micocin X® concentration of 0.5% (w/w) in low sodium bologna. However, combination with other antimicrobial treatments is recommended due to the fact *L. monocytogenes* is a versatile microorganism and can grow at different pH, a_w values and temperatures.

Regarding the diversity and abundance of meat microbial communities, the culture-independent analysis by Ion Torrent showed no difference between treatments with or without Micocin X®, which in turn, allowed inferring there was an interaction between bologna and bacteriocin, thereby affecting the inhibitory activity of Micocin X® on spoilage bacteria. When comparing the effect of sodium concentration on the microbial populations it was observed that the percentage of sequences of the order *Bacillales* was greater on 3% NaCl meat product due to the fact LAB are sensitive to higher concentrations of sodium, as demonstrated in chapter 3.

5. GENERAL DISCUSSION

The food industry has recent, increased interest in producing low salt products because an excess sodium intake is well known to be the major cause of high blood pressure levels, hypertension and other diseases such as cardiovascular disease and kidney failure.

In the meat industry, sodium is used as a hurdle technique and the effects or implications that its reduction may have on the microbiological safety and quality in food has received little attention.

The overall objective of this project was to investigate the microbial ecology and dynamics of spoilage and pathogenic microorganisms in low-sodium sliced bologna product without addition of a sodium replacement. Indiscriminate reduction of NaCl from processed foods may not only enable pathogen growth and survival, it may also allow accelerated microbial spoilage of the product, causing a negative economic impact to producers, distributors, retailers, and consumers.

In the first part of this project (Chapter 3), sliced cooked bologna was formulated with 1%, 2% and 3% NaCl, inoculated with spoilage bacteria, and stored under vacuum and aerobic conditions. The spoilage microorganisms in this project consisted of microorganism commonly isolated from cooked ready-to-eat meats, such as *Brochothrix thermosphacta*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides* and *Pseudomonas fluorescens*. In terms of sodium levels, 3% NaCl was chosen as a highest sodium level, 2% NaCl served as a control sodium level since commercial cooked ready-to-eat meat products are formulated at this sodium concentration, and 1% was chosen as the lowest concentration due to the fact lower values may negatively-affect product cohesiveness and processing characteristics, in particular, the firmness of the slices which were approximately 3 mm in thickness.

The effect of product sodium concentration on microbial growth was followed using culture-dependent techniques for a minimum period of 30 days. In this study it was shown that sodium reduction from 2% to 1% on sliced cooked bologna stored at 4°C did not affect bacterial spoilage rates. Based on a preliminary study, it was demonstrated that temperature is a major

factor regarding food deterioration; previous research has also demonstrated that growth rate and lag phase are highly temperature-dependent (Taormina, 2010). On the other hand, this study demonstrated that packaging conditions serve to control microbial growth; oxygen availability in vacuum-packaging conditions had a significant influence on microbial growth rate. The microbiological analysis of uninoculated bologna by the plate count technique revealed the presence *P. fluorescens, Br. thermosphacta* and lactic acid bacteria as part of the initial product microbiota. When cooked bologna was stored under aerobic conditions, all microbial groups increased in number faster compared to that seen under vacuum-packaged conditions. However, by the end of the storage under either packaging treatment the analyzed bacteria attained high viable counts. The number of spoilage bacteria was initially retarded over the first days of storage by the restriction of air under vacuum-packaged conditions. However, by day 9, viable counts increased and *Br. thermosphacta* and lactic acid bacteria, particularly homo- and heterofermentative LAB such as *Lactobacillus, Leuconostoc* and *Lactococcus*, were the main bacterial spoilage groups detected under this condition.

Lowering sodium concentrations in ready-to-eat meat formulae is considered to increase the risk of growth of pathogens such as *L. monocytogenes*. This bacterium can survive at low pH, water activity and low sodium concentration, and has motivated the meat processing industry to develop innovative ideas to deliver products higher in quality, safer and more natural while containing less preservatives. This was the basis for conducting the second study where cooked bologna formulated with 1 and 3% NaCl was produced with and without Micocin X®, which is a cell-free preparation containing three bacteriocins produced by *Carnobacterium maltaromaticum* UAL307.

In a previous study conducted by Miller (2010), Micocin X® was applied to control the growth of spoilage bacteria in pasteurized liquid whole eggs, and extended the product's shelf life to 5 weeks. However, there are no other research studies reporting on its efficacy in meat products.

The results of study 2 shows that a concentration of 0.5% of Micocin X® did not significantly-reduce spoilage bacterial counts. In contrast, in the presence of Micocin X®, *L. monocytogenes* did not increase in numbers over the first 15 days of storage but after approximately 20 days Listeria seems to adapt, affecting its sensitivity to Micocin X®.

Ion Torrent culture-independent analysis showed the potential of using next generation sequencing platforms in meat research and allowed the evaluation of microbial community of food and food environments in greater detail. Characterization of microbial community structure of meat and processing environment could help our understanding of the processes of cross-contamination through the meat processing chain, and could also provide more detailed information that would allow the development of new practices to ensure the production of a wholesome product. During this project, it was difficult to consistently extract DNA concentration from all treatments. PCR amplicons were purified using Qiagen's purification kit (Qiagen Sciences, Germantown, MD, USA) and it was unsuccessful as yield and quality were low.

6. GENERAL CONCLUSIONS

This study was conducted to investigate the dynamics of spoilage and pathogenic microorganisms in low sodium, sliced, cooked bologna product by molecular and traditional culture-based methods. The matrix system was formulated with 1, 2 and 3% which corresponded to a sodium content of 525 mg/100 g, 919 mg/100 g and 1071 mg/100 g serving, respectively.

Results obtained from culture-dependent technique demonstrated that sodium reduction can be applied in our specific bologna recipe without the addition of salt replacements. The growth of spoilage bacteria on cooked slice bologna under aerobic and vacuum packaging conditions indicated that salt concentration not only affects microbial growth rate but also the type and numbers of bacteria.

The approach to study the spoilage-related bacteria by using Ion Torrent revealed that plate count techniques might not be enough to characterize the microbial community. As was observed in this study, meat ecology is complex and many of the microorganisms identified haven't been reported previously on cooked ready-to-eat meats and their identification by culture-dependent techniques would have been difficult since by using plate culture, a particular population of microorganisms is selected for. As generally accepted, about 99% of the microorganisms from any environment are not cultivable under laboratory conditions using standard plate-culturing techniques, as many of them require specific conditions and nutrients, or they cannot be grown in isolation within the laboratory setting. Ion Torrent high throughput sequencing allowed a more comprehensive description of the microbial communities that were present in multiple samples in a rapid and economic manner.

Redox potential is an important intrinsic factor of meat systems that can be controlled or utilized in order to provide a safe product. In this study, the implementation of redox measurements to monitor spoilage bacteria growth was shown to be a complementary tool that can be applied to assess products shelf-life, as the metabolic activity (and utilization of oxygen) of microorganisms will lead to the creation of reducing local environments. However, the use of microelectrodes in sliced meat systems is not recommended as an indicator of food spoilage at

this time since difficulties were encountered in taking accurate and reproducible measurements. It was thought that maintaining intimate and consistent contact between the microelectrode and the test surface (bologna) was the primary issue, and although the method is highly-sensitive, it is also highly sensitive to slight differences in the meat microenvironment.

Micocin X® was added into the meat blend in order to determine its potential as antimicrobial agent in low sodium ready-to-eat meat products. Results demonstrated that it may be of particular use in controlling the growth of *L. monocytogenes*. It was found to be relatively ineffective against the general spoilage microflora inhabiting the meat system. However, it is important to mention that it may exert a bacteriostatic effect, which for instance, could function to supplement control of various microbes based on application of multiple antimicrobial hurdles.

Future research is required to study the effect of Micocin X® in combination with other antimicrobial techniques, such as EDTA, Lysozyme or nisin, under different packaging conditions or in different meat matrices. It would also be interesting to study microbial changes throughout the storage period-course using Ion Torrent sequence analysis in order to get more detail information about microbial dynamics during the storage period. Improvements to DNA recovery and avoiding biases in PCR might also be a targeted objective for future culture-independent work.

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